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23. The method of claim 1 wherein said symptoms further includes elevated glucose levels.

24. The method of claim 22 wherein said symptoms further includes elevated glucose levels.

Remarks

Claims 1-15 and 22-24 are pending in the present application. Claims 16-21, directed to compositions, have been cancelled in an effort to expedite the allowance of the instant application. Claims 23-24 are new. Support for the amendment to the claims can be found throughout the specification, examples and claims in particular, in the specification at pages 3, 8 and pages 26, lines 25-26 and page 27, the last two paragraphs and in the discussion of Figure 10. Applicants consider the claims allowable in their presently amended form. The Examiner maintained his rejection of the previously filed claims 1-22 are unpatentable as being obvious under 35 U.S.C. ¶103 over the cited references. For the reasons which are presented in the sections which follow, it is respectfully submitted that the instant application is now in condition for allowance and such action is earnestly solicited.

The courtesy of the Examiner Hui's telephonic interview with Dr. Martha Belury and the undersigned attorney on the afternoon of May 11, 2004 is respectfully acknowledged. Applicants present amended claims in this action in conformity with the discussions during that interview in order to seek allowance of the instant claims. Support for the patentability of the claims is also presented in the attached declaration of Dr. Martha Belury, a co-inventor of the subject matter of the instant application.

The §103 Rejection

The Examiner has rejected previously filed claims 1-22 under 35 U.S.C. §103 as being

obvious over de Boer, et al., U.S. patent no. 5,518,751 ("de Boer"), in view of Cook, et al., U.S. patent no. 5,554,646 ("Cook"). It is the Examiner's position that de Boer teaches that CLA in food compositions such as milk are useful in treating disorders such as diabetes. The Examiner points to column 1, lines 35-43 in support of that view. The Examiner recognizes that de Boer does not teach particularly that CLA is useful in a method of treating diabetes, the specific conjugated linoleic acids claimed or the amount of CLA of the present invention. The Examiner cites Cook for teaching a method of adding CLA compounds into animal feed to reduce fat in an animal and that specific isomers of octadecadienoic acid may be included in the conjugated linoleic acid.

From the teachings of the cited art the Examiner concludes that it would have been obvious to employ CLA in a method of treating diabetes and that it would have been obvious for one of ordinary skill in the art at the time the invention was made to incorporate about 1 mg to about 10,000mg/kg of body weight of the *trans,cis*-9,11-octadecadienoic acid, *cis,cis*-9,11-octadiendioic acid or *trans,cis*-10,12-octadecadienoic acid into a milk composition product useful in a method of treating diabetes. The Examiner further argues that one of ordinary skill would have been motivated to employ CLA in a method of treating diabetes because de Boer, et al. clearly teaches unsaturated fatty acids, including CLA, are useful in treating disorders such as diabetes. It is the Examiner's conclusion, therefore, that one of ordinary skill would have reasonably *expected* that CLA would have been useful in a method of treating diabetes. Applicants respectfully traverse the Examiner's rejection.

It is respectfully submitted that the Examiner's arguments with respect to the presently pending claims are not cogent for the following reasons. First, de Boer is an ambiguous reference as to the treatment of all of the indicated disease states with all of the indicated fatty acids. Thus, in the first instance, de Boer does not make out a cogent case that CLA can be used to treat the symptoms of type II diabetes mellitus of the present invention, when the type of diabetes is not mentioned by de Boer and a number of fatty acids may be used with equal effectiveness to treat the disclosed disease states, which list includes diabetes. As stated in the attached Belury declaration at paragraph 23, diabetes is a disease state represented by at least three different disease states: Type I diabetes mellitus, Type II diabetes mellitus and diabetes insipidus. In addition, as is set forth in that declaration, it is shown that conjugated linoleic acid, alone among the fatty acids listed, is particularly effective in treating type II diabetes mellitus symptoms. Dr. Belury points out that in her own research conjugated linoleic acid has exhibited a dramatic effect on type II diabetes mellitus symptoms and linoleic acid showed essentially no effect on these same symptoms, whereas the teachings of de Boer suggest that either of these two fatty acids can be used to treat diabetes with apparent equal effect. The present invention is unexpected from the teachings of de Boer. Thus, de Boer cannot be cited for the proposition and there is no suggestion in de Boer that conjugated linoleic acid is a particularly effective treatment for type II diabetes mellitus symptoms, namely glucose intolerance and elevated plasma insulin and glucose. The present invention is therefore non-obvious over the teachings of de Boer.

Cook Does Not Obviate the Deficiencies of de Boer

Turning to the disclosure of Cook, this reference discloses a method of using CLA to reduce the body fat of animals, including humans. Although this reference supports the view that CLA may be used to reduce body fat and increase protein, primarily in meat animals, there is absolutely no disclosure or suggestion of the use of CLA in the treatment of type II diabetes mellitus symptoms. There is absolutely no mechanism discussed in Cook for the effects CLA exhibits in animals regarding reducing body fat. Cook provides absolutely no motivation for

treating the symptoms of type II diabetes mellitus namely glucose intolerance and elevated plasma insulin and glucose and is respectfully believed to be inapposite to the present invention.

It is respectfully submitted that a combination of de Boer and Cook does not disclose or suggest the present invention. There is no disclosure or suggestion that conjugated linoleic acid, alone among the fatty acids disclosed by de Boer, provides an effective treatment for type II diabetes mellitus symptoms, as explained by Dr. Belury in the attached declaration, an unexpected result. Whereas de Boer suggests that a number of fatty acids may be used to treat diabetes, conventional research evidences that conjugated linoleic acid, alone among the fatty acids disclosed can be used to effectively and dramatically to treat type II diabetes mellitus symptoms as claimed.

In the present application, there is simply no cogent basis upon which to suggest that the prior art taught the use of CLA for the treatment of type II diabetes mellitus symptoms. The disclosure in de Boer suggests that all unsaturated fatty acids can be used, whereas conventional research evidences this is not the case. While the disclosure in de Boer does not disclose or suggest the presently claimed invention, the remaining art cited, Cook, does not disclose or suggest the treatment of diabetes *at all.* In particular, not only does Cook fail to mention diabetes, Cook fails to even mention a single symptom of diabetes set forth in the claims. Consequently, Applicants respectfully submit that the present invention is patentable over the disclosure of de Boer, in view of Cook.

The Examiner has also maintained his rejection of claims 1-22 under 35 U.S.C. §103 as being obvious over Semenkovich and Heinecke, *Diabetes*, 1997, 46:327-334 ("Semenkovich"), in view of Steinhart, *Journal of Chemical Education*, 1996, 73(12):A302 and Cook (see above).

In sum, the Examiner cites Semenkovich for teaching that most diabetic patients die from macrovascular complications and that oxidative modification of lipoproteins in diabetic patients

is enhanced, with this being one of the major risks for developing cardiovascular complications (macrovascular complications) in diabetic patients. Semenkovich is also cited for teaching that antioxidants are potent inhibitors of lipoprotein lipid peroxidation and thereby reduce the lipoprotein oxidation products and cytotoxicity caused by those products. The Examiner acknowledges that Semenkovich does not expressly teach the employment of CLA in a method to treat diabetes or the symptoms of diabetes or the specific isomers of octadecaienoic acid or amounts of CLA.

The Examiner cites Steinhart for teaching CLA as a natural antioxidant. Cook is cited for teaching a method of adding CLA to animal feed.

From the disclosures of Semenkovich, Steinhard and Cook as set forth in the office action, the Examiner contends that the present invention is obvious and therefore, unpatentable. Applicants respectfully traverse the Examiner's rejection. A combination of these references in no way teaches or suggests that CLA was known or would have been expected to be a particularly effective treatment for type II diabetes mellitus symptoms.

Semenkovich is a reference which describes the relationship between diabetes and atherosclerosis, noting that in the vast majority of cases, individuals which exhibit symptoms of diabetes do not, in fact, develop premature vascular disease. See page 327 of Semenkovich, second column. In addition to the somewhat limited connection between diabetes and atherosclerosis is the fact that the mechanism for development of premature vascular disease is not particularly well understood. Indeed the title of the Semenkovich article is ***"The Mystery of Diabetes and Atherosclerosis*** *Time for a New Plot*." While Semenkovich teaches that antioxidants may be useful in addressing issues associated with oxidized lipoproteins in the development of atherogenesis, there is absolutely no disclosure or suggestion that CLA *in particular* would be useful in the treatment of *diabetes*. Indeed, CLA is not even mentioned. Rather, the antioxidant of choice in Semenkovich is ascorbate (page 332), a particularly potent

antioxidant, which has significantly different physicochemical characteristics compared to CLA. Even a suggestion in Semenkovich that antioxidants (ascorbate) might be useful in reducing lipoprotein oxidation products and therefore, may play a beneficial role in limiting atherogenesis, does not evidence that CLA as an antioxidant could play such a role. See, for the example, the previously enclosed Abstract of Berliner and Heinecke, *Free Radic. Biol. Med.*, 1996, 20(5):707-727 ("Berliner"), cited in Semenkovich (note *64)*, which clearly indicates that the mechanism of oxidation of lipoprotein is promoted by several different systems, including protein-bound metal ions, thiols, reactive oxygen intermediates, lipoxygenase, peroxynitrite and myeloperoxidase. While Semenkovich may suggest the generic use of antioxidants to treat macrovascular disease in those limited number of diabetic patients in which such a condition occurs, there is absolutely no suggestion in Semenkovich that CLA should be used to treat atherosclerosis or that antioxidants should be used to treat diabetes, and in particular type II diabetes mellitus or its symptoms. Indeed, there is absolutely no disclosure in Steinhart of type II diabetes mellitus or its treatment using CLA. *Noted here is the fact that even Semenkovich acknowledges that only a limited number of diabetic patients actually are at risk for macrovascular disease, most likely based upon some genetic predisposition.*

A limited disclosure, Semenkovich cannot possibly be read to suggest the use of CLA as a treatment modality for diabetes and in particular, type II diabetes mellitus symptoms. It cannot even be fairly said that Semenkovich suggests CLA as a treatment modality for macrovascular disease, because it is not clear from the disclosure of Semenkovich (which cites Berliner) or from Berliner itself, that CLA would be a particularly effective antioxidant, given the lack of understanding of the oxidative process in producing such a condition and the fact that ascorbate, a particularly potent antioxidant, is disclosed. Semenkovich is clearly a deficient reference.

Steinhart does nothing to cure the deficiencies of Semenkovich, other than to suggest that CLA *may be* (obvious to try) useful to treat atherosclerosis, which occurs in a limited number of diabetic patients. See Semenkovich at page 327. Steinhart discloses generally, that CLA is a

natural antioxidant, which has important uses in the limitation of carcinogenesis and in certain limited instances *perhaps*, atherogenesis. Steinhart further discloses, on page 4 of the article, that when rabbits and hamsters were fed cholesterol-supplemented diets, animals which also received CLA had lower levels of total and LDL (i.e., "bad") cholesterol in their blood and developed less atherosclerosis in their aortas. Thus, Steinhart, at best, teaches that CLA *may be* (i.e., obvious to try) useful in limited instances in reducing the tendency of hypercholesterolemia to develop further into atherosclerosis. However this reference does not suggest the use of CLA to treat atherosclerosis, merely that it would be *obvious to try* CLA in such treatment. Obviously, from the disclosure in Steinhart further research would be required. Thus, combining the disclosures of Semenkovich with Steinhart *at best*, merely suggests that CLA may be useful to treat atherosclerosis, in instances where hypercholesterolemia, and in particular, high LDL levels, are present. Thus, the disclosure of Steinhart provides at best an *obvious to try* approach to the treatment of atherosclerosis. There is absolutely no expectation in Steinhart that CLA actually could be used to treat atherosclerosis, given that the disclosure is directed to laboratory test animals, i.e., rabbits and hamsters, fed a high cholesterol diet. Thus, Steinhart provides nothing more than the possibility of the use of CLA to treat atherosclerosis, not a suggestion that CLA actually could treat atherosclerosis. As set forth in the attached declaration of Dr. Martha Belury, almost eight years after the publication of Steinhart and Semenkovich, CLA is still not recognized as a treatment for atherosclerosis, in the presence or absence of diabetes. Indeed, the previously enclosed paper and abstract, Khosla and Fungwe, *Current Opinions in* Lipidology, 12(1), pp. 31-34 (February, 2001) indicates that even in 2001, it was not clear that CLA was actually useful for treating atherogenesis and the animal models and related research do not permit such a conclusion. Khosla, at page 33, second column. Applicants note that the Khosla reference is not prior art to the present application and was published after the filing date of the present application. Nonetheless, this reference was presented to provide evidence as to whether or not one of ordinary skill at the time of the filing date of the present application would have considered the use of CLA in the treatment of atherosclerosis in general, obvious or not. Obviously, one of ordinary skill in the art could not have considered such a method obvious,

inasmuch as such a method was not obvious five years later in 2001 as evidenced by the Khosla, supra, paper, or almost eight years later as evidenced by the attached declaration of Dr. Belury (paragraph 33), a person intimately familiar with current research trends in CLA.

Thus, it is not obvious that CLA is useful for the treatment of atherosclerosis and it is certainly not obvious that CLA is useful for treating the symptoms of type II diabetes mellitus, inasmuch as Steinhart does not even mention type II diabetes mellitus or the claimed symptoms. To the extent that the Examiner has made an obviouness rejection, that rejection is an *obvious to try* rejection, which fails in the instant case to render the present invention obvious. Given that the art still does not yet recognize CLA in the treatment of atherosclerosis *almost eight years after the disclosure of Steinhart,* this evidences that Steinhart does not and cannot obviate the deficiencies of Semenkovich. Moreover, there is absolutely no disclosure or suggestion in Steinhart that CLA is useful for the treatment of type II diabetes mellitus or its symptoms (e.g., improving glucose tolerance and lowering plasma insulin and glucose levels), or even that the type of atherosclerosis associated with diabetes or type II diabetes mellitus would be treated by CLA, given the mechanistic complexicity of such a disease. In short, Steinhart does not even obliquely suggest that CLA can be used to treat symptoms of type II diabetes mellitus and certainly in no way obviates the deficiencies of Semenkovich.

It is noted that a coincident side effect of improving glucose control and reducing glycated cellular products according to the present invention *may be* that the oxidation of LDL is reduced. One could speculate that this might be of benefit to a person by reducing athersclerosis/cardiovascular disease (ASCVD) risk. Reducing the oxidation of LDL is the putative teaching of Semenkovich and Steinhart upon which the Examiner relies to posit that CLA is a viable treatment for atherosclerosis in diabetic patients. However, the speculated benefit the Examiner posits from the combined teachings is not recognized by the art as an actual treatment and the art fails to recognize CLA as a *bona fide* treatment of atherosclerosis or to reduce atherogenesis. The results so far do not support such a treatment. See, Khosla, supra,

and the Belury declaration. Thus, it is not obvious to use CLA in the treatment of atherosclerosis. Moreover, in addition to the fact that CLA is not recognized in the art as a treatment of atherosclerosis is that there is absolutely ***no*** (ie., ***zero***) evidence that reducing oxidation of LDL would be a viable treatment for type II diabetes mellitus or its symptoms (e.g., glucose intolerance or elevated plasma levels of insulin and/or glucose) in people with type II . Thus, the combined disclosures of Semenkovich and Steinhart do not and cannot make out a cogent rejection of the instant invention.

Turning to Cook, Cook does not obviate the deficiencies of the combined disclosures in Semenkovich and Steinhart in failing to suggest the present invention. Cook has been discussed supra, and that discussion is referenced here. Cook teaches that CLA may be used in the feed of animals in order to reduce body fat and increase protein mass. There is no disclosure in Cook that CLA should be used to treat the symptoms of type II diabetes mellitus, nor does the reduction in body fat and increase in body protein in Cook have any relevance to the treatment of diabetes or its symptoms. The teachings of Cook are really irrelevant to the symptoms of diabetes, and without even an oblique reference to the treatment of diabetes in Cook, Cook cannot be fairly seen to obviate the deficient disclosures of Semenkovich and Steinhart.

Thus, there is absolutely no teaching or suggestion that one can glean from the combined teachings of Semenkovich, Steinhard and Cook that evidences that CLA is useful or should be used to treat type diabetes mellitus or its symptoms.

For the above reasons, Applicant respectfully asserts that the claims set forth in the present amendment are now in compliance with 35 U.S.C. Applicants respectfully submit that the present application is now in condition for allowance and such action is earnestly solicited.

Applicant has cancelled six claims. No fee is therefore due for the presentation of this amendment. A petition for a two month extension of time is enclosed. A check for the

appropriate fee is enclosed. Small entity status is claimed for the present application.

Please credit any overpayment or charge any additional fee due to Deposit Account No. 04-0838.

Respectfully submitted,

COLEMAN SUDOL SAPONE, P.C.

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Dated: June 4, 2004

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I hereby certify that this correspondence is being deposited with the U.S. Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450, on June 7, 2004.

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Henry D. Coleman (Reg. No. 32,559)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S) : Vanden Heuvel, et al.
SERIAL NO. : 09/555,987
FILED : September 11, 2000
FOR : Methods and Compositions for Treating Diabetes
GROUP ART UNIT : 1617
EXAMINER : Sang Ming Hui

DECLARATION OF DR. Martha Belury

I, Martha Belury declare as follows:

1. I am a co-inventor of the subject matter of the above-referenced patent application.

2. I am a citizen of the United States of America.

3. In 1987, I received a B.S. degree in Nutrition/Dietetics from the University of Texas in Austin, Texas.

4. In 1992, I received a Ph.D. degree in Biological Sciences from the University of Texas in Austin, Texas.

4. Since 1988, I have been involved in the investigation and research on the chemical basis of nutrition and its impact on a variety of health concerns, including diabetes. I have done extensive research on the role of conjugated linoleic acid in nutrition and its impact on human disease states since 1992.

5. I am presently the Carol S. Kennedy Endowed Professor of Nutrition at The

Ohio State University in Columbus, Ohio. I have held this position since September, 2002.

6. Since February, 2004, I have been an Associate Adjunct Professor of Medicine in the Department of Internal Medicine at The Ohio State University in Columbus, Ohio.

7. Since September, 2002, I have been a Tenured Associate Professor in the Department of Human Nutrition at The Ohio State University in Columbus, Ohio.

8. From May 2000, I have been an Adjunct Associate Professor in the Department of Foods and Nutrition at Purdue University, West Lafayette, Indiana.

9. From September, 2000 through September, 2002, I was an Associate Member of the Department of Molecular Medicine at Northwest Hospital, Seattle, Washington.

10. From July, 1998 through May, 2000, I was an Associate Professor with Tenure in the Department of Foods and Nutrition at Purdue University, West Lafayette, Indiana.

11. From August, 1994 through July, 1998, I was an Assistant Professor in the Department of Foods and Nutrition at Purdue University, West Lafayette, Indiana.

82. From August, 1992 through July, 1994, I was an Assistant Professor in the Department of Health and Human Development, Montana State University, Bozeman, Montana.

93. From March, 1988 through August, 1992, I was a Graduate Research Assistant at the University of Texas, in Austin, Texas.

104. I have received numerous awards and honors for my scientific work

including: Scientific Advisor to the Council for Women's Nutrition Solutions (CWNS), National Cattleman's Beef Association, from 1999 until the present; Speaker's Bureau, National Cattleman's Beef Association on Various Topics, including Bioactive nutrients such as conjugated linoleic acids, and the Nutritional modulation of processes such as type 2 diabetes, obesity and cancer from 1998 until the present; the E.L.R. Stokstad Award for Outstanding Research, American Society Nutritional Sciences (ASNS), April, 2000; Outstanding Young Professional Award for 1999, Texas Exes in Human Ecology, University of Texas, Austin, Texas; Most Influential Teacher, College of Education, Health and Human Development, Montana State University, 1993; Pre-Doctoral NIH Training Grant in Toxicology, University of Texas, Austin, Texas, 1990-1992; and the Society of Toxicologists of the Southwest Ted Reynolds Graduate Center Aware, M.D. Anderson-Science Park Center Research Center in 1991.

115. I am or have been a member of a number of Grant Review Panels for the United States Department of Agriculture, National Institutes of Health, American Institute for Cancer Research, U.S. Army Breast Cancer Grants, the American Chemical Society Institutional Grants, the Indian Elks Cancer Research Grants, Purdue Cancer Center, as well as various International Research Organizations, including the Dairy Farms of Canada, Health Research Board, Dublin, Ireland, NIH MBRS Grant Proposal, International Food Society, among others.

16. I have participated as a symposia chair or organizer for a number of scientific meetings, including several in 2001-2003 being directed specifically to conjugated linoleic acid. In addition, I am an Ad Hoc Journal Reviewer for various national and international peer-reviewed journals including: *Journal of Lipid Research, Cancer Research, Cancer Letters, American Journal of Physiology, Lipids, Journal of Nutrition, Journal of Nutritional Biochemistry, Nutrition and Cancer, American Journal of Clinical Nutrition, Journal of Food Science, and the Journal of the American Diabetic Association.*

17. I am a member of a number of Professional organizations including the American Diabetes Association, America Society of Nutritional Sciences, American Society for Biochemistry and Molecular Biology, American Oil Chemists' Society, OSU Nutrition (OSUN) Interdepartment Nutrition Program, as well as a number of Professional Association Committees.

18. I have published over 40 scientific papers and I have published numerous reviews and chapters in the area of nutrition, many of which were directed to conjugated linoleic acid ("CLA") and its mechanism of action. In addition, I have given numerous scientific/research lectures at national science meetings/symposia.

129. I am familiar with and am a co-inventor of the subject matter of United States patent application serial number 09/555,987, which claims are directed to the treatment of type II diabetes mellitus in a diabetic patient comprising administering an effective amount of conjugated linoleic acid to that patient. Thus, the present invention relates to the unexpected finding that the use of conjugated linoleic acid will favorably influence glucose metabolism in diabetic patients and can be used to treat type II diabetes mellitus in a diabetic patient. I understand that the Examiner has rejected the in the patent application as being obvious over a combination of references which the Examiner contends in combination, teach that conjugated linoleic acid can be used to treat diabetes.

20. Diabetes is typically characterized as one of several diseases. Diabetes may refer to diabetes insipidus, which is a disease of chronic excretion, causes dehydration and extreme thirst in those diabetic individuals. Diabetes mellitus, another form of diabetes is generally of two types: Type I diabetes mellitus, which is an insulin-dependent diabetes mellitus, and Type II diabetes mellitus, also known as non-insulin-dependent diabetes mellitus. Type I diabetes mellitus is a metabolic disease in which carbohydrate utilization is reduced and which is caused by an absolute or relative deficiency of insulin. It is characterized by hyperglycemia,

glycosuria, water and electrolyte loss, ketoacidosis and coma. Type II diabetes mellitus, the subject of the present invention, generally occurs in people who are over 35, where glucose tolerance is low and where plasma insulin and glucose levels are elevated.

21. From reading the office action dated January 7, 2004, I understand that the Examiner has essentially made two arguments that the previously filed claims were unpatentable as being obvious over certain references cited by the Examiner. In the first rejection, the Examiner contends that the teachings of de Boer, et al., U.S. patent number 5,518,751 ("de Boer"), in view of Cook, et al., U.S. patent number 5,554,646 ("Cook") render the claimed invention unpatentable as being obvious. In the second rejection, the Examiner cites the teachings of Semenkovich and Heinecke, *Diabetes*,1997, 46:327-334 ("Semenkovich"), in view of Steinhart, *Jounral of Chemical Education,* 1996;73(12):A302 and Cook as rendering the previously presented claims unpatentable as being obvious.

22. I have reviewed the disclosures of de Boer and Cook. De Boer is primarily directed to a method for including certain unsaturated fatty acids into milk products to prevent them from becoming rancid. The major discussion of de Boer is really irrelevant to the presently claimed invention. The only relevant disclosure in de Boer is that which appears in de Boer in column 1 at lines 35-42. That disclosure is set forth below in italics:

> *An important reason for enriching milk or milk powders with fats containing a high percentage of unsaturated fatty acids or strongly unsaturated fatty acids is to preventor reduce cardiovascular diseases, atopies, rhumatic disorders or diabetes. In particular, such products contain a high percentage of oleic acid, linoleic acid which may or may not be conjugated, α-linolenic acid and unsaturated C_{20} and C_{22} fatty acids.*

23. Although this passage in de Boer is quite ambiguous to me, I take it to mean that all of the cited fatty acids which are set forth in de Boer may be used to prevent or reduce

diabetes of any type. My own research evidences that of all of the fatty acids cited by the relevant passage in de Boer, the only one which has a significant positive impact on type II diabetes mellitus in diabetic patients is conjugated linoleic acid.

24. In particular, my own research has evidenced that there is a dramatic difference in the impact that conjugated linoleic acid has compared to linoleic acid in treating the claimed symptoms of type II diabetes mellitus. Under my supervision and control, rigorous investigations have been conducted on the ability of conjugated linoleic acid and simple linoleic acid to produce effects in patients with Type II diabetes mellitus. My own research, as well as contemporary research by other investigative groups, evidences that linoleic acid which is not conjugated has little or no effect on the claimed symptoms of type II diabetes mellitus, whereas the effect of conjugated linoleic acid on type II diabetes mellitus is *dramatic.* These data suggest that the effect of fatty acids other than the conjugated dienoic fatty acids of CLA, are independent of effects on hyperglycemia, the clinical diagnostic symptom of type 2 diabetes. Unlike findings with non-conjugated dienoic fatty acids, including those disclosed by de Boer, the conjugated dienoic fatty acids (CLA) produced lower fasting insulin, lower fasting glucose, and improved glucose tolerance in the Zucker diabetic fatty (ZDF; fa/fa) rat model. See , attached as Exhibit 2.

25. We have since shown that supplementation of people with type 2 diabetes with CLA reduced fasting glucose which is unlike the effects seen when safflower oil supplements which contain the non-conjugated fatty acid, linoleic acid (one of a number of fatty acids the Examiner has argued is taught by de Boer to treat diabetes) are used. The CLA produced dramatic effects, whereas the effects of linoleic acid were negligible. Thus, the actions of CLA and linoleic acid in type II diabetic mellitus individuals were markedly different.

26. The relationship of dietary fat quality to type 2 diabetes has been investigated

in several recent intervention studies. These studies have found a role for oleic acid (18:1n9) for favorably altering lipid profiles in normal subjects as well as type 2 diabetes individuals. See Meyer, et al., *Diabetes Care* 24: 1528-1535 (2001); Lichtenstein and Schwb, *Atherosclerosis* 150:227-243 (2000); Sameron, et al., *Am J Clin Nutr*, 73:1019-1026 (2001); *N Engl J. Med* 346: 393—402 (2002); and Lermer-Garber, et al., *Diabetes Care* 17:311-315 (1994), references enclosed. These studies further evidence that the effect of non-conjugated fatty acids, including those disclosed by de Boer, are *independent of the effects on hyperglycemia*, the clinical diagnostic symptom of type 2 diabetes. In addition, the chemical and physiological differences for conjugated linoleic acid compared to linoleic acid and other non-conjugated polyunsaturated fatty acids are numerous and have been reviewed in several of my recent recent reviews. See, Belury, *Annu Rev Nutr*, 22:505-531 (2002); Belury, *J Nutr*, 132:2995-2998 (2002); and Belury, onjugated linoleic acid supplementation modulates glucose and lipid homeostais in subjects with type 2 diabetes mellitus. (References enclosed). Invited chapter in: Conjugated linoleic acid, Volume II: Review and Physiological Mechanisms of Action. Ed. J-L Sebedio, AOCS Press, Champaign, Il, in press. The differences range from effects in carcinogenesis, metabolism, obesity/energy balance, and type 2 diabetes. This would explain the marked difference CLA exhibits on the symptoms of type II diabetes mellitus compared to the negligible impact seen when the non-conjugated fatty acids of de Boer are used.

27. Thus, contemporary research regarding the biological activity of unsaturated fatty acids such as those disclosed in de Boer relative to the activity of conjugated linoleic acid in treating the symptoms of type II diabetes mellitus as claimed stands in complete contrast to the teachings of de Boer which indicates that both linoleic acid and conjugated linoleic acid (as well as other unsaturated fatty acids such as oleic acid) may be used to prevent or "reduce" diabetes. My research, as well as other contemporary research, has shown that conjugated linoleic acid, *alone* among the fatty acids disclosed by de Boer, has a substantial favorable impact on type II diabetes mellitus symptoms as claimed when administered in effective amounts to type II

diabetes mellitus patients. This finding is completely unexpected from the teachings of de Boer.

28. Turning to the teachings of Cook, I believe that these teachings are irrelevant to the present invention. Cook adds nothing to the teachings of de Boer. Cook's teachings are directed to the use of CLA to decrease body fat and increase the protein content primarily of meat animals to be slaughtered for food. There is absolutely no teaching in Cook that CLA may be used to treat diabetes *of any kind,* especially in particular, type II diabetes mellitus or its symptoms as claimed. Moreover, there is absolutely no disclosure in Cook as to how CLA may be reducing body fat and increasing the body protein content. I note that the use of CLA to decrease body fat and increase body protein in animals is readily distinguishable from the use of CLA to lower plasma levels of triglycerides and/or fatty acids, which may be elevated in type II diabetes mellitus. Indeed, many individuals who have low body fat content have high plasma triglyceride and free fatty acid levels, and many individuals with high body fat have normal triglyceride and free fatty acid levels. There is no causal connection between these conditions. The mere fact that Cook teaches a reduction in body fat and increase in body protein in normal animals does not suggest in any way the administration of CLA in a type II diabetes mellitus patient to lower elevated plasma triglyceride and/or free fatty acid levels. Nor do the teachings of Cook have any connection to any of the other symptoms of type II diabetes mellitus which are treated by the present invention. Consequently, I believe that it is not obvious to use CLA to treat type II diabetes mellitus from the combined teachings of de Boer, in view of Cook.

29. I understand that the Examiner also has rejected the previously filed claims 1-22 based upon his view that the claimed subject matter is obvious over the teachings of Semenkovich, in view of Steinhart and Cook. From my reading of the January 7, 2004 office action, I understand that the Examiner cites Semenkovich for teaching that oxidative modification of lipoproteins is elevated in diabetic patients, and that antioxidants may be used to reduce lipoprotein oxidation products and cytotoxicity caused by those products, with a result

that atherosclerosis is reduced. The Examiner notes that Semenkovich does not teach the use of conjugated linoleic acid to treat *any form of* diabetes or the symptoms of diabetes. The Examiner has cited Steinhart for teaching that conjugated linoleic acid is a natural oxidant. Finally, the Examiner has cited Cook for teaching the addition of conjugated linoleic acid to animal feed to reduce body fat in the animal eating the feed. From a combination of the teachings of these references, the Examiner has submitted that the invention is obvious. I disagree.

30. As the Examiner notes, neither Semenkovich nor Steinhart teach the use of conjugated linoleic acid to treat diabetes in any form. The Examiner is citing this combination of references to suggest that because CLA is an antioxidant and Semenkovich postulates that strong antioxidants may be used to reduce atherosclerosis, it is obvious to use CLA to treat atherosclerosis in diabetic patients. I disagree. Although Steinhart does disclose that conjugated linoleic acid is an antioxidant, this reference neither discloses conjugated linoleic acid for the treatment of the symptoms of diabetes, nor does this reference even mention diabetes in general, or more particularly, type II diabetes mellitus, the symptoms of which are treated by the presently claimed method. The only relevant disclosure in Steinhart to the present invention is the passage on page 4 which relates to the potential use of conjugated linoleic acid for reducing atherosclerosis. This passage states:

> "Rabbits and hamsters are frequently used to study diet-induced atherosclerosis. When rabbits and hamsters were fed cholesterol-supplemented diets, animals who also received CLA had lower levels of total and LDL ("bad") cholesterol in their blood and developed less atherosclerosis in their aortas."

31. Steinhart does not teach or suggest that CLA may be used to treat type II diabetes mellitus. In fact, Steinhart does not even teach the treatment of atherosclerosis. What Steinhart teaches in the above passage is that CLA appears to influence the amount and quality of

cholesterol in laboratory test animals fed a high cholesterol diet and that those animals showed a reduction in atherosclerosis. Steinhart therefore teaches at best that further testing may be conducted to determine what impact CLA may have on atherosclerosis. A recent article, Khosla and Fugnwe, "Conjugated linoleic acid: effects on plasma lipids and cardiovascular function", *Current Opinin in Lipidology*, 2001, 12:31-34, which was previously cited to the Examiner, puts the teachings of Steinhart in perspective. This reference at page 32, in the bottom of the first and the top of the second column, indicates that the studies on the use of CLA to treat atherosclerosis in humans which were conducted until 2001, the date of that publication, were *"inconclusive in predicting how CLA will behave in man"*. Page 32, second column, lines 9-10. The reference continues that "*there is at present no evidence in support of the anti-atherogenic effect of CLA.*" Page 32, second column, lines 11-12. I am currently very active in conjugative linoleic acid research and I note that almost eight years after the teachings of Steinhart, CLA is still not recognized as a treatment for atherosclerosis. To this date, I do not have any expectation that CLA can be used to treat atherosclerosis, especially given the amount of research conducted to date in this area.

32. The teachings of Cook are deficient for the reasons which have been previously discussed. The reduction in body fat in animals caused by CLA as taught by Cook is irrelevant to the treatment of type II diabetes mellitus symptoms using CLA according to the present invention. Cook does not mention diabetes, type II diabetes mellitus symptoms or any of the claimed symptoms of the present invention. I therefore believe that the combined teachings of Semenkovich, in view of Steinhart and Cook do not teach or suggest that CLA can be used to treat type II diabetes mellitus or the symptoms of type II diabetes mellitus as claimed.

33. I further declare that all statements made herein of my own personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: June 5, 2004 Martha Belury

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DIETARY CONJUGATED LINOLEIC ACID IN HEALTH: Physiological Effects and Mechanisms of Action[1]

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Key Words CLA, gene expression, lipid metabolism, fatty acid, nutrition

■ **Abstract** Conjugated linoleic acid (CLA) is a group of polyunsaturated fatty acids found in beef, lamb, and dairy products that exist as positional and stereo-isomers of octadecadienoate (18:2). Over the past two decades numerous health benefits have been attributed to CLA in experimental animal models including actions to reduce carcinogenesis, atherosclerosis, onset of diabetes, and body fat mass. The accumulation of CLA isomers and several elongated/desaturated and β-oxidation metabolites have been found in tissues of animals fed diets with CLA. Molecular mechanisms of action appear to include modulation of eicosanoid formation as well as regulation of the expression of genes coding for enzymes known to modulate macronutrient metabolism. This review focuses on health benefits, metabolism, and potential mechanisms of action of CLA and postulates the implications regarding dietary CLA for human health.

CONTENTS

[1]Abbreviations: CLA, conjugated linoleic acid; PPAR, peroxisome proliferator-activated receptor; ZDF, Zucker diabetic fatty (fa/fa).

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INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids that exist as positional and stereo-isomers of conjugated dienoic octadecadienoate (18:2). The predominant geometric isomer in foods is the c9t11-CLA isomer (36, 69) [also called "rumenic acid" (59)], followed by t7,c9-CLA, 11,13-CLA (c/t), 8,10-CLA (c/t), and the t10c12-CLA isomer (36). The three-dimensional stereo-isomeric configuration of CLA may be in combinations of cis and/or trans configurations. CLA is found in foods such as beef and lamb, as well as dairy foods derived from these ruminant sources (20, 38, 69). Synthetically prepared oils of CLA are composed of an isomeric composition somewhat different than isomers found naturally in foods. A method of preparation for synthetic CLA oil has traditionally relied on an alkaline-catalyzed reaction using linoleate as substrate. The isomeric composition of synthetic CLA oil with ~90% purity that is prepared using linoleate (18:2cis9cis12) as a substrate is: c9t11/t9c11-CLA (~42%) and t10c12-CLA (~43%), with c9c11-CLA, c10t12-CLA, t9t11/t10t12-CLA, 7,9-CLA, 8,10-CLA, and 11,13-CLA comprising minor amounts. In addition, this 90% pure CLA contains residual substrate (0.5% linoleate) plus some oleate (~5.5%) and unidentified fatty acid (4.0%). Aside from preparation, the purification of synthetic compositions of CLA oil and individual isomers warrants attention. Due to high cost and/or lack of availability, very few studies conducted in vivo have used highly purified isomers or naturally extracted CLA oil. Thus, for the most part, studies conducted in experimental animals and humans to demonstrate the physiological effects of CLA are attributable to the synthetic mixture of isomers (predominantly c9t11-CLA and t10c12-CLA) (Figure 1). Little has been done in vivo to determine the activity and mechanisms of isomers other than these two. Except where noted, the remainder of this review focuses primarily on studies using the synthetic mixture of CLA oil in vivo or purified isomers isomers in cultured cells in vitro.

HEALTH PROPERTIES OF CONJUGATED LINOLEIC ACID

Numerous physiological properties have been attributed to CLA including action as an antiadipogenic, antidiabetogenic, anticarcinogenic, and antiatherosclerotic agent (Table 1) [reviewed in (12, 13)]. In addition, CLA has effects on bone formation and the immune system as well as fatty acid and lipid metabolism and gene expression in numerous tissues (8, 12, 31, 41, 64, 85).

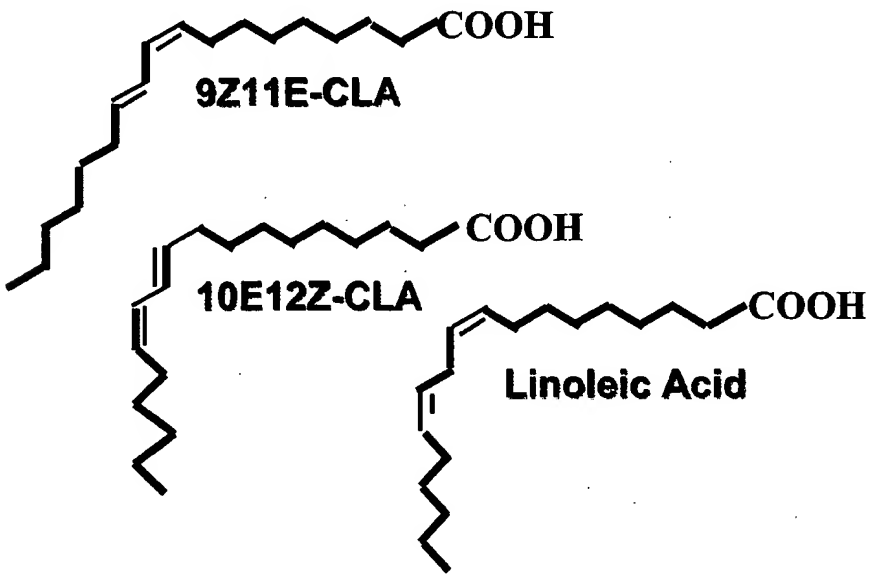

Figure 1 Structures of cis9trans11-CLA, trans10cis12-CLA and linoleic acid (18:2cis9cis12). These isomers are also referred to as c9t11-CLA or 9Z11E-CLA and t10c12-CLA or 10E12Z-CLA, respectively.

Conjugated Linoleic Acid Reduces Adipose Tissue

A plethora of data demonstrates that CLA modulates body composition, especially by reducing the accumulation of adipose tissue, in experimental animals. In mice, rats, pigs, and humans, dietary CLA reduces adipose tissue depots (29, 80, 100, 101). Early work to demonstrate the adipose-mass lowering effect of CLA was performed in growing mice where postweanling mice (6 weeks old) were fed a diet containing 1.0% CLA for 28–32 days (80). Total adipose tissue mass was reduced by over 50% compared with mice fed a control diet (without CLA). Further work demonstrated that the dietary CLA reduction of adiposity could be sustained in mice even after CLA was removed from the diet (81). Subsequent studies in nonobese mice demonstrated that some depots of fat mass [especially retroperitoneal and epididymal white adipose tissue masses (26, 108) and brown adipose tissue (108)] might be more sensitive to CLA-mediated reductions. In contrast to findings in nonobese rats, obese Zucker rats (100), but not Zucker diabetic fatty (ZDF) rats (41), exhibited an adipose-enhancing effect of dietary CLA (100). Long-term feeding of CLA (1.0% CLA for 8 months) appears to have a lipodystrophic effect in female C57BL/6J mice, leading to complete ablation of brown adipose tissue, reduced leptin, a hormone known to regulate feed intake, increased fat accumulation in the liver, and eventual development of insulin resistance (108).

TABLE 1 Physiological properties of conjugated linoleic acid

Major function	Physiological model	References
Body composition	↓ Adiposity in chicks, mice, and rats	24, 80, 100
	↑ Adiposity in obese Zucker rats	100
	↓ Adiposity in ZDF rats	41
	↓ Adiposity is isomer specific (t10c12-CLA)	84
	↓ Adiposity in human subjects	101, 105
	↔Adiposity in human subjects	116
Diabetes	↓ Onset of diabetes in ZDF male rats	41
	Aids in the management of metabolic parameters in human subjects with type 2 diabetes	M.A. Belury, unpublished data
	↓ Insulin sensitivity in mice	108
Carcinogenesis	↓ Chemically induced mammary carcinogenesis in rats	46
	↓ Chemically induced mammary carcinogenesis in rats by either c9t11-CLA or synthetic CLA	47
	↓ Chemically induced mammary carcinogenesis in rats regardless of level of fat or esterification of CLA (in triglyceride) vs. free fatty acid	45, 52
	↓ Growth of transplantable breast cancer tumor cells in nude mice	42,109
	↓ Growth of transplantable prostate cancer tumor cells in nude mice	19
	↓ Stages of chemically induced skin tumorigenesis in mice	11, 39
	↓ Chemically induced colon carcinogenesis in rats	65
	↔ Carcinogenesis in Min mice	87
	↓ Chemically induced forestomach	40
Atherosclerosis	↓ Atherosclerotic plaque formation in hamsters	113
Bone formation	↓ Eicosanoid production	64
Immune system	↓ Eicosanoid and histamine production	104, 112
	↑ Onset of lupus in mouse model	115

↓, decreases; ↑, increases; ↔, no effect; CLA, conjugated linoleic acid; ZDF, Zucker diabetic fatty.

In addition to some indications that the effect of CLA on adiposity may be dependent on preexisting adiposity, the effects of CLA on body composition may be gender specific. Male rat pups are more responsive to dietary CLA, resulting in reduced adipose and increased muscle mass compared with female pups. In addition, there appeared to be an isomer-specific effect of CLA on adiposity: t10c12-CLA was much more effective at lowering adipose tissue mass than the c9t11-CLA isomer in mice (84). In addition, t10c12-CLA appeared to be the effective isomer for modulating gene expression in cultured 3T3-L1 preadipocytes (21). The ability

of CLA to reduce adipose tissue mass occurs regardless of food intake or fat level (6.5–20.0%) in mice, so feed efficiency may be improved (9, 26, 80). In fact, CLA reduces leptin in rats (13) and humans (M.A. Belury, unpublished data).

PUTATIVE MECHANISMS OF CONJUGATED LINOLEIC ACID REDUCTION OF ADIPOSITY Mechanisms of how CLA reduces adiposity in lean animals, and perhaps in humans, may revolve around pathways that regulate energy expenditure (111). In fact, feeding a semipurified diet containing CLA (1.0%) to male AKR/J mice for 6 weeks resulted in significantly increased metabolic rates and reduced nighttime respiratory quotients (111). When Std ddY mice were gavaged with CLA (5 ml/kg body weight), the increased oxygen consumption was associated with significantly increased oxidation of fat, but not carbohydrate (79). The cellular basis of the enhanced oxidation of lipids is not thought to require peroxisomal β-oxidation (25, 70). Because the hormones noradrenaline and adrenaline were also significantly higher in mice gavaged with CLA (79), the data suggest that CLA enhances sympathetic nervous activity that leads to increased energy metabolism and eventual reduction of adipose tissue mass.

The ability of CLA to reduce adipose tissue mass has also been linked with induction of adipocyte apoptosis and/or differentiation. Induction of apoptosis by CLA occurred in preadipocyte cultures (33). In addition, female mice fed a diet with 1.0% CLA for 8 months exhibited increased apoptosis in brown and white adipose tissues (108). The induction of apoptosis of adipose tissues was associated with induction of TNF-alpha and uncoupling protein-2. Uncoupling protein-2 is a member of the mitochondrial uncoupling protein family and functions to "uncouple" the transfer of electrons over the inner mitochondrial membrane, resulting in thermal dissipation of energy as heat in place of adenosine triphosphate. The induction of uncoupling protein-2 in muscle by CLA was also demonstrated in ZDF (fa/fa) rats (91) and therefore may be a mechanism of increased energy expenditure in mice fed CLA (110). A diet with CLA (1.0%) did not induce uncoupling protein mRNA in muscle of mice, although the same mice exhibited increased energy expenditure. Therefore, it was concluded that uncoupling protein-2 may not be a significant mediator of effects of CLA on energy utilization and adiposity.

Induction of markers of differentiation of adipose tissue by dietary CLA was first shown in vivo in male ZDF (fa/fa) rats fed 1.5% CLA for 2 weeks (41). In this study the adipocyte lipid binding protein (ap2), a marker of adipocyte differentiation (95), was increased approximately fivefold over levels in rats fed a control diet (without CLA). In vitro studies using 3T3-L1 preadipocytes demonstrate mixed effects of CLA: At least one study showed that CLA enhanced differentiation of 3T3-L1 preadipocytes (as assessed by lipid accumulation in cells) (93), whereas another study showed that CLA inhibited differentiation (17). Because of the limitations of using a programmed preadipocyte, interpreting these data and extrapolating them to an in vivo setting is difficult. In fact, it was recently shown that timing of

CLA treatment in cultured preadipocytes is critically important to determining the differential effects of CLA on inducing differentiation (applied early) or inhibiting differentiation (applied after 3 days of programmed culturing) (33).

In addition to modulating apoptosis and differentiation, CLA may reduce adipose tissue mass by minimizing accumulation of triglycerides in adipocytes. t10c12-CLA inhibits activity of lipoprotein lipase in vivo (83). Because lipoprotein lipase aids in the incorporation of fatty acids into triglycerides in adipocytes, these data suggest that the adipose-lowering effects of t10c12-CLA result from reduced uptake of fatty acids into adipocytes. In fact, triglycerides and glycerol levels were reduced in 3T3-L1 cells (33). Notably, the addition of linoleate could partially restore the content of triglycerides in cultured preadipocytes (17).

CONJUGATED LINOLEIC ACID HAS DIFFERENTIAL EFFECTS ON BODY FAT IN HUMANS In adult humans the ability of CLA to lower adipose tissue mass has been demonstrated in some (15, 101, 105) but not all (72, 116) studies. For example, when overweight or obese human subjects were supplemented with CLA (3.4–6.0 g/day) for 12 weeks, a significant reduction of fat mass was observed (15). However, in people consuming 3.0 g/day for 12 weeks, no benefit was observed for body weight or adiposity (116). More recent studies have demonstrated that CLA supplementation reduces body weight, leptin, and body adiposity in people [(101, 105); M.A. Belury, unpublished data]. It is likely that dose, duration (short- or long-term), and the isomeric composition of CLA each impact the ability of CLA to affect obesity in humans. In addition, how strain/species-, age-, and sex-specific effects of various isomers of CLA affect adipose tissue accumulation, either in obese humans or those seeking to prevent adipose gain, is yet to be determined.

Conjugated Linoleic Acid Modulates Metabolic Parameters of Type 2 Diabetes

There are numerous risk factors for the development of type 2 diabetes including the presence of impaired glucose tolerance, ethnicity, age, gender, and genetics. Central to all of these risk factors is obesity (27). Based on the fact that CLA reduces adiposity in experimental animals, we designed a study to elucidate the role of CLA in the development of type 2 diabetes in male ZDF (fa/fa) rats. Male ZDF rats were fed semipurified diets containing no CLA (control), 1.5% CLA, or the antidiabetic thiazolidinedione drug, troglitazone (0.02%) for two weeks. Rats fed the CLA or thiazolidinedione diet exhibited significantly reduced ($p < 0.05$) fasting glucose, insulinemia (41), triglyceridemia, free fatty acid levels, and leptinemia (13) compared with control rats. In addition, dietary CLA induced aP2 mRNA, a marker of adipose differentiation, in vivo (41). A recent study using a similar protocol, but with various sources of CLA, demonstrated that a mixture of CLA isomers induces adipose-lowering effects in ZDF rats and enhances glucose uptake into muscle of ZDF rats (91). In contrast, butter enriched with c9t11-CLA

exerted little, if any, ability to reduce glucose tolerance, lower adipose tissue, or modulate glucose uptake into muscle. The authors (91) speculated that the t10c12-CLA isomer may be the biologically active isomer in delaying the onset of diabetes reported earlier (41). However, the role of specific CLA isomers in delaying the onset and/or reducing the severity of type 2 diabetes is yet to be measured directly.

Whereas CLA reduces fasting insulin in diabetic animals, it modestly increases fasting serum insulin in nondiabetic swine (103), mice (108), and humans (71). Because fasting insulin may be used as a surrogate marker for insulin resistance, these data suggest that CLA reduces insulin sensitivity under a normoglycemic state. In agreement, after long-term feeding (8 months) of a CLA-diet, an induction of insulin resistance was observed in C57Bl/6J male mice (108). CLA-induced insulin resistance was associated with lipodystrophy. The impact and significance of CLA for reducing insulin sensitivity and/or altering lipodystrophy for people who are normoglycemic is unknown.

Because CLA was able to delay the onset of diabetes in the ZDF rat model, CLA as an aid in the management of type 2 diabetes in humans was examined (M.A. Belury, unpublished data). A double blind, randomized study to determine the effect of daily supplementation with CLA or placebo (safflower oil) on metabolic parameters of diabetes was conducted. Subjects with type 2 diabetes were provided with supplements with CLA or placebo, instructed to maintain a healthy diet using the Food Guide Pyramid, and asked not to change their diet or activity habits for the 8-week intervention period. CLA supplementation (6.0 g CLA/day) significantly decreased fasting blood glucose, plasma leptin, body mass index, and weight. Low density lipoprotein levels significantly increased, but less in the CLA-supplemented group than in the placebo group. In addition, body fat (%) was modestly decreased ($p < 0.08$) in subjects supplemented with CLA. Fasting insulin, HbA_{1c}, triglycerides, cholesterol, and high density lipoprotein were not significantly affected by CLA. According to 3-day diet records, energy intake was not significantly different between groups at baseline or throughout the study. We concluded that supplementation with CLA for 8 weeks could be associated with favorable alterations of several metabolic parameters of subjects with type 2 diabetes. Further work is needed to determine the therapeutic potential of CLA in the management of type 2 diabetes.

Dietary Conjugated Linoleic Acid Inhibits Carcinogenesis

Dietary CLA inhibits numerous cancer models in experimental animals. In particular, it inhibits skin tumor initiation and forestomach neoplasia (39,40). In addition, the synthetic mixture of CLA isomers inhibits chemically induced skin tumor promotion as well as mammary and colon tumorigenesis when added to semisynthetic diets (11,46,65). Importantly, the inhibitory effect of CLA on mammary carcinogenesis is independent of type or level of fat in the diet and occurs in a dose-dependent manner (45,46). When transplanted into nude mice, growth of mammary (109) or prostate (19) cancer cell lines was significantly reduced if animals were fed a diet with CLA (1.0%). The inhibition of chemically induced

mammary carcinogenesis occurred whether CLA was fed as a free fatty acid or triglyceride (52). Furthermore, the 9,11-CLA and 10,12-CLA isomers appear to be equally active in inhibiting mammary carcinogenesis in rats (54).

Although the inhibitory role of CLA is convincing, not all studies consistently demonstrate that CLA inhibits carcinogenesis. In fact, CLA was unable to alter the growth of transplanted prostate (96) and breast (114) cancer cells in some studies and did not reduce tumorigenesis in an intestinal model of colon carcinogenesis using the Apc Min mouse model (87). No studies report that CLA enhances tumorigenesis. In contrast to effects of CLA on carcinogenesis, the n-6 and n3 fatty acids, linoleate (18:2c9c12) and eicosapentaenoate (20:5n3), have differential effects (from no effects to potent enhancing or inhibitory effects) depending on the tumor model and tissue studied [reviewed in (34)]. Therefore, the ability of CLA to inhibit multiple models of carcinogenesis appears to be specific for this group of fatty acids. Furthermore, some of the mechanisms and functions of CLA are likely to be unique among polyunsaturated fatty acids [reviewed in (7, 43); (51)].

PROPOSED MECHANISMS OF INHIBITION OF CARCINOGENESIS BY CONJUGATED LINOLEIC ACID Efforts have been made to elucidate the mechanistic role of CLA in modulating carcinogenesis by determining the effects on the stages of carcinogenesis known as initiation, promotion, and progression [reviewed in (12)]. In fact, the anticarcinogenic property of CLA was first identified during the initiation stage of the mouse skin multistage carcinogenesis model (39), where the stages of initiation, promotion, and progression are operationally separable (28). In this initial study a lipid fraction extracted from fried ground beef was topically applied to mouse skin prior to initiation of the carcinogen, 7,12-dimethylbenz(a)anthracene. Tumor yield (average number of tumors per mouse) after 16 weeks of promotion with phorbol ester was inhibited by approximately 45%. In a manner independent from its antiinitiator activity, CLA was then shown to inhibit tumor promotion (11). Mice were fed semipurified diets containing various levels of synthetically prepared CLA (5% corn oil plus 0%, 0.5%, 1.0%, or 1.5% CLA) after initiation and for the duration of promotion with the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate (35 weeks). Mice fed 1.5% CLA exhibited an inhibition of tumor yield of ~30% compared with mice fed control diets (containing no CLA). In chemically induced rat mammary carcinogenesis the stages of initiation and promotion are not readily separable. Nevertheless, when fed before or after carcinogen treatment (postinitiation), dietary CLA inhibited carcinogenesis as well (45, 52).

Whereas a great deal of evidence demonstrates that dietary CLA inhibits the initiation and promotion stages of carcinogenesis, the role of CLA in the progression stage of carcinogenesis has not been comprehensively addressed. In transplantable tumor models, dietary CLA reduced the growth rates of cells when implanted in vivo (19, 109). In addition, at least one study demonstrated that CLA (0.5–1.0%) inhibited the growth of transplanted mammary cancer cells to form secondary tumors in mice (42). Furthermore, the CLA-responsive chemically induced mammary carcinogenesis model (46) is a model for human breast cancer ductal carcinomas in

situ. Therefore, data showing that CLA inhibits tumorigenesis of this model are consistent with the possibility that CLA reduces metastasis resulting from breast cancer. However, no studies have addressed the role of CLA in the prevention of metastatic cancer. It is critical to understand how CLA modulates malignant tumor formation and metastasis because the growth of secondary tumors is the major cause of morbidity and mortality in people with cancer.

CONJUGATED LINOLEIC ACID MODULATES NUMEROUS EVENTS DURING TUMOR PROMOTION In order to elucidate the anticarcinogenic mechanisms of CLA, early work focused on events associated with initiation. As an antiinitiator, CLA may modulate events such as free radical–induced oxidation, carcinogen metabolism, and/or carcinogen-DNA adduct formation in some tumor models [reviewed in (7)]. In recent years, attention has focused on elucidating the mechanisms of action of CLA that inhibit carcinogenesis during promotion, particularly in the mammary and skin carcinogenesis models (52). The promotion stage involves the clonal expansion of initiated cells to form a benign tumor. This stage of carcinogenesis represents a premalignant state in which tumors arise from cells that have increased cell proliferation, reduced programmed cell death (or apoptosis), and/or dysregulated differentiation. In cultured cells, CLA reduced proliferation of mammary tumor cells in vitro (30, 99) and in vivo (53). In cultured mammary epithelial cell organoids, CLA (64 μM) or c9t11-CLA (128 μM) inhibited cell growth (48). In vivo, rats that were initiated with methylnitrosourea and then fed a diet with CLA (1.0%) exhibited reduced proliferation of terminal end bud and lobuloalveolar bud structures of mammary epithelium (using histochemical analyses of bromodeoxyuridine staining) (106). Importantly, the terminal end bud is the site of tumor formation for both rat and human breast cancer. The inhibition of proliferation by CLA was accompanied by a reduction in density of the terminal end bud (106). More recently, the reduction of cell proliferation in terminal end bud structures by dietary CLA was accompanied by reduced levels of two cyclins known to regulate the cell cycle, cyclin D1 and cyclin A (47). These data suggest that CLA modulates molecular signaling events that impact the cell cycle, ultimately regulating cell proliferation.

Studies to determine the role of CLA in modulating cell proliferation in models of carcinogenesis other than mammary carcinogenesis have been elusive. In contrast to findings in mammary carcinogenesis, there was no relationship between dietary CLA and markers of cell proliferation of mouse epidermis (hyperplasia, ornithine decarboxylase activity, or c-myc mRNA expression) (56). These data suggest that inhibition of skin tumor promotion by CLA may not occur through inhibition of cell proliferation of mouse epidermis. In rat liver, increasing levels of dietary CLA (0.5–1.5%) increased cell proliferation in diethylnitrosamine-induced focal lesions (68), demonstrating that the ability of CLA to reduce cell proliferation may be tissue-specific and/or tumor model–specific. In other models of carcinogenesis, the mechanistic role of CLA in modulating cell proliferation has not been identified.

As a counterbalancing event in promotion, apoptosis offers protection to carcinogenesis via programmed death of cancer cells. Dietary CLA induced apoptosis in numerous tissues including mammary (48), liver (68), and adipose (108) tissues and in cultured mammary epithelial cells (50). In mammary tissue initiated with methylnitrosourea, dietary CLA induced apoptosis of cells in the terminal end bud and in premalignant lesions known as intraductal proliferation lesions (48). In these studies, CLA induction of apoptosis was associated with a reduction of bcl-2, a signaling protein known to suppress apoptosis. These data suggest that CLA may inhibit promotion by inducing signaling events leading to enhanced apoptosis.

CLA induces markers of differentiation in the noncancer model, adipose tissue (41, 93). Therefore, it is possible that CLA inhibits carcinogenesis via induction of differentiation. In fact, the finding that CLA fed during the time of mammary gland development and maturation has long-lasting protective effects on mammary carcinogenesis (52, 106) suggests that the role of CLA in protecting against mammary carcinogenesis may be, in part, by modulating tissue differentiation.

Dietary Conjugated Linoleic Acid Modulates Atherosclerotic Plaque Formation

There is a growing body of evidence that CLA reduces atherosclerotic plaque formation in experimental animals. When CLA (0.5 g/rabbit/day) was added to a hypercholesterolemic diet and fed to rabbits for 12 weeks, serum triglycerides and low density lipoprotein cholesterol levels were significantly reduced compared with rabbits fed a diet without CLA (60). Importantly for heart disease risk, aortas of rabbits fed the CLA-containing diet exhibited less atherosclerotic plaque formation. In a subsequent study, hamsters were fed a diet with or without CLA designed to induce hypercholesterolemia (76). The diet with CLA (1.0%) reduced plasma total cholesterol, non–high density lipoprotein-cholesterol, and early aortic atherosclerosis relative to a diet with linoleate (113). In a similar model in hamsters fed a hypercholesterolemic diet, c9t11-CLA, the sole CLA isomer in the diet, had no effect on plasma lipids (37). Because dietary CLA was associated with significantly reduced formation of dienes, it was concluded that the ability of CLA to reduce aortic plaque formation could be due to changes in low density lipoprotein oxidative susceptibility. In contrast to protective effects of CLA on atherosclerotic plaque formation in rabbits and hamsters, CLA induced the formation of aortic fatty streaks in C57Bl/6 mice fed an atherogenic diet (75).

The effects of CLA on thrombotic properties of blood cells have been studied in cultured platelets in vitro and in human subjects. In cultured platelets, CLA, c9t11-CLA, and t10c12-CLA inhibited collagen- or arachidonate-induced platelet aggregation (107). These findings were associated with reduced production of the proaggregatory cyclooxygenase products of ^{14}C-arachidonate, ^{14}C-thomboxane-A2, and ^{14}C-thromboxane-B2. In human subjects supplemented with CLA (3.9 g/day) or placebo (sunflower oil) for 93 days, there were no differences in platelet aggregation or prothrombin time (14).

Because CLA appears to exert differential effects on lipid profiles as well as atherogenic markers in various animal models, further work is needed to demonstrate the mechanisms of CLA for the prevention of atherosclerosis and its role in reducing cardiovascular disease risk in humans.

Other Health Properties of Conjugated Linoleic Acid

As a group of fatty acids, CLA displays numerous benefits in experimental animals. In addition to reducing the onset and/or severity of carcinogenesis, obesity, diabetes, and atherosclerotic plaque formation, CLA may affect the rate of bone formation in rats (63). However, combined with an omega-3-rich menhaden oil, dietary CLA exerted no further beneficial effects on bone formation. In support of a positive role of CLA in bone formation, rat pups exposed to CLA (0.5%), either in utero or during the first 7 days of life, had significantly longer tail lengths (a measure of skeletal growth) compared with pups fed a diet without CLA (88).

CLA modulates several events in immunity that may revolve around modulating eicosanoid formation. Arachidonate-derived eicosanoids, derived through cyclooxygenase and lipoxygenase pathways, are produced by numerous types of immune cells and are thought to regulate cytokine synthesis and inflammation. Initial studies demonstrated that CLA protects against *Escherichia coli*-induced weight loss in chicks and mice (23) and reduces histamine-induced prostaglandin-E2 production in Guinea pig trachea (112). However, in a model of the autoimmune disorder, lupus erythematosis, dietary CLA accelerated the onset of proteinuria (115). In this same study, CLA was protective against the development of end-stage symptoms of lupus. These data suggest CLA may have differential effects on disorders involving immunity since CLA exacerbates early stage, but delays late stage, symptoms of lupus.

In rats assigned to a diet with CLA (1.0%), levels of leukotriene-B4 and leukotriene-C4 in spleen and lung were reduced (104). The inhibition of leukotriene levels was associated with significantly reduced non-stimulated histamine. However, in humans supplemented with CLA (3.9 g/day) for 93 days, no apparent alterations in eicosanoids (e.g., prostaglandin E-2, leukotriene B-4) or cytokines (e.g., interleukin-1 beta or tumor necrosis factor alpha) were observed (57). The discrepancy of data between different models of immune function and between animal and human models argues that further study of the role of CLA in immunity is needed. In particular, it is important to determine the modulation of various immune disorders, especially autoimmunity and immunodeficiency by individual isomers, dosages, and duration of dietary CLA.

METABOLISM OF CONJUGATED LINOLEIC ACID

It is well established that when provided in the diet or as a supplemental oil, CLA isomers accumulate in tissues of animals and humans [reviewed in (7); (55)]. In addition, isomers of CLA are readily metabolized in vivo via multiple

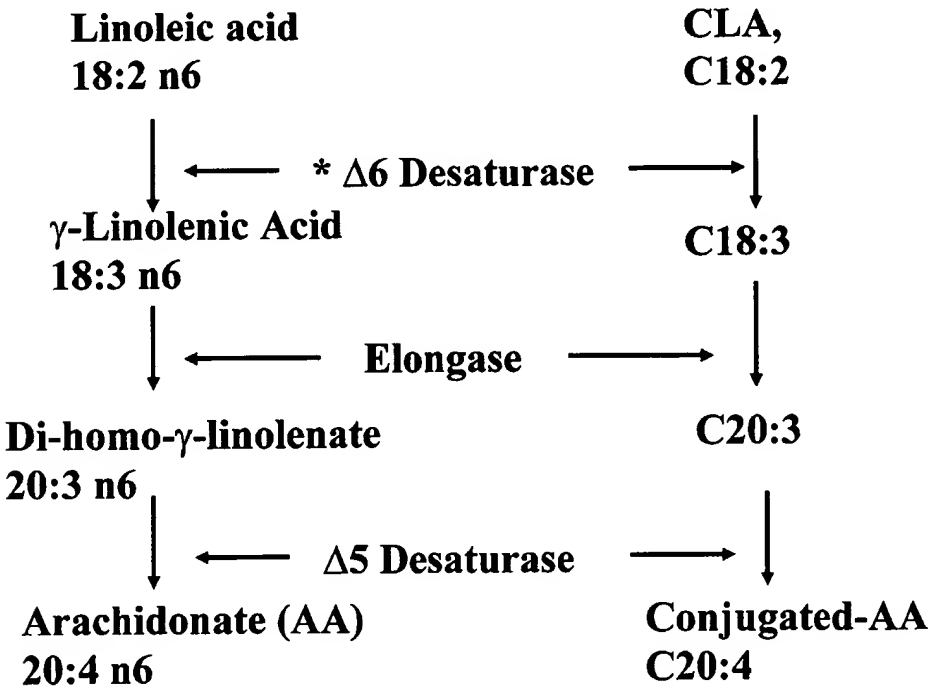

Figure 2 Pathway for desaturation and elongation of CLA. *Δ6 desaturase, rate limiting step. AA, arachidonate.

metabolic pathways. Elongated and desaturated metabolites of CLA (Figure 2) (e.g., conjugated-18:3, conjugated-20:3, and conjugated-20:4) have been identified in the liver (3, 4, 49) and mammary tissue (2) of rats and adipose tissue and sera of humans (1; M.A. Belury, unpublished data) (Table 2). In fact, ^{14}C-CLA is metabolized to the same extent (to form ^{14}C-conjugated-18:3) as ^{14}C-linoleic acid when compared in an enzymatic assay using an hepatic isolate of Δ6 desaturase enzyme (8). In addition to forming desaturase and elongase products, CLA is readily oxidized to β-oxidation products (16:1 and 16:2), presumably from peroxisomal β-oxidation of downstream elongated/desaturated metabolites of CLA (1, 49).

The role of metabolites of CLA to modulate tissue responses such as adipose tissue mass, glucose sensitivity and/or carcinogenesis is pending further investigation. The biological activities of CLA metabolites are hampered by the lack of availability of purified metabolites (e.g., conjugated gamma-linolenate, 18:3; conjugated eicosatrienoate, 20:3; and conjugated eicosatetraenoate, 20:4) for use in cell culture and in vivo feeding experiments. As an alternative approach, a 19-carbon conjugated fatty acid, conjugated nonadecadienoate (19:2), which is assumed to be metabolized to different downstream products of CLA, was fed to

TABLE 2 CLA isomers and metabolites

Isomers in foods[a]	Δ6 Desaturase	Conjugated anabolites formed elongase	Δ5 Desaturase		
c9t11-CLA	c6c9t11-octadecatrienoate (18:3)	c8c11t13-eicosatrienoate (20:3)	c5c8c11t13- eicosatetraenoate (20:4)		
	—	c8c11t13-eicosatrienoate (20:3)		Rat Liver Rat Mammary 2 Human Plasma Human Serum	1, 3, 97; M. Belury and S. Banni, Unpublished data
t7,c9-CLA	N.D.[b]	N.D.	N.D.	—	
11,13-CLA (c/t)	N.D.	N.D.	N.D.	—	
8,10-CLA (c/t)	N.D.	N.D.	N.D.	—	
t10c12-CLA	c6t10c12-octadecatrienoate (18:3)	c8t12c14-eicosatrienoate (20:3)	c5c8c11t13-eicosatetraenoate (20:4)	Rat Liver Rat Mammary 2 Human Plasma Human Serum	1, 3, 97; M. Belury and S. Banni, Unpublished data
	—	t12c14-eicosadienoate (20:2)			

[a]Listed in order of prevalence in most foods (36).

[b]N.D., not determined.

mice (0.3% of diet) (83). Mice fed nonadecadienoate exhibited significantly lower adipose tissue accumulation (by ~81%) compared with mice fed a control diet. In contrast, mice fed a diet with CLA exhibited a 25% reduction in adipose tissue. In 3T3-Li preadipocytes conjugated nonadecadienoate and CLA had similar efficacy on reducing heparin-releasable lipoprotein lipase and lipid accumulation (17). Because of the difference in hydrocarbon chain length between CLA and conjugated nonadecadienoate, it is likely that metabolites, including those from Δ6 desaturase, are different. Therefore, the authors concluded that the Δ6 desaturase metabolites of CLA may not be important for the alterations in gene expression induced by CLA. It is likely that the biological activities of some metabolites may overlap with biological properties of the parent conjugated octadecadienoates (18:2), whereas others may not.

CONJUGATED LINOLEIC ACID MODULATES LIPID METABOLISM

Conjugated Linoleic Acid Modulates Fatty Acid Composition of Phospholipids and Alters Eicosanoid Formation

Like most other dietary polyunsaturated fatty acids, isomers and metabolites of CLA are readily incorporated into phospholipid and neutral lipid fractions of numerous tissues (8, 40, 45, 46, 73). In some studies incorporation of CLA into phospholipids of cultured cells occurs in a manner that is similar to linoleate (66 , 74). When radiolabeled tracers were used to study the kinetics of ^{14}C-CLA uptake into keratinocytes or hepatoma cells, ^{14}C-CLA was incorporated to the same extent and at a similar rate as ^{14}C-linoleate. In addition, the level of incorporation of ^{14}C-CLA and ^{14}C-linoleate into epidermal phospholipid and neutral lipid fractions was similar (66). However, a recent study of rats fed a diet containing CLA-rich butter (and linoleate) showed that accumulation of CLA and linoleate into rat liver is not similar (3). In fact, CLA preferentially accumulated in neutral lipids (~79%) with less incorporation into phosphatydilcholine (~10%), the major phospholipid of liver cells. In contrast, linoleate accumulated preferentially in phosphatydilcholine (~50%), with less in neutral lipids (~17%).

Of the two main isomers studied, c9t11-CLA accumulates to a higher extent than t10c12-CLA in tissue phospholipids of liver (3, 8), skin (56), and bone (64) of experimental animals. Furthermore, in neutral lipids of mammary (44) and muscle (31), c9t11-CLA accumulates to a greater extent than the t1012-CLA. The higher level of c9t11-CLA may be due to either preferred incorporation into tissues and/or more rapid metabolism of the t10c12-CLA isomer. In support of the latter, we have found that human subjects supplemented with CLA (6.0 g/day; ~37% c9t11-CLA and ~39% t10c12-CLA) for 8 weeks show significantly higher accumulation of c6t10c12-CLA, the Δ6 desaturase metabolite of t10c12-CLA compared with the amount of metabolite formed from c9t11-CLA (e.g., c6c9t11-CLA) in serum (M.A. Belury & S. Banni, unpublished data).

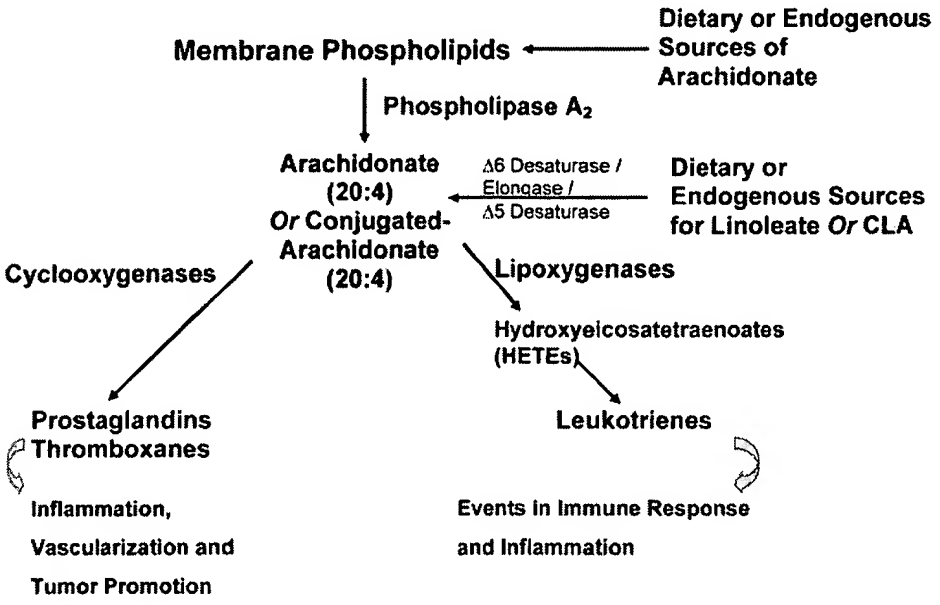

Figure 3 General schematic pathway for eicosanoid synthesis from arachidonate.

Based on findings from a number of laboratories, it is likely that one mechanism for the ability of CLA to exert many of its physiological functions (e.g., carcinogenesis, diabetes, obesity, immunity, bone formation, and platelet aggregation) is by modulating the accumulation of arachidonate in phospholipids, resulting in a reduced arachidonate pool and reduced production of downstream eicosanoid products (Figure 3). In fact, the role of CLA to reduce cyclooxygenase products (e.g., prostaglandin-E2, prostaglandin F2alpha) has been demonstrated in vivo in bone and macrophages (64, 104) but not small intestine tissue from Min mice (87) or spleen from rats (104). In addition, we have found that phorbol-ester-induced prostaglandin-E2 is reduced in the epidermis of mice fed CLA (56) and in cultured keratinocytes in vitro (67). Furthermore, dietary CLA reduced accumulation of the lipoxygenase products leukotriene-B4 and leukotriene-C4 in spleen and lung (104) but not ^{14}C-hydroxyeicosatetraenoic acid (^{14}C-12-HETE) in cultured human platelets (107). It appears that CLA modulation of eicosanoid production is tissue specific.

The mechanism of how CLA reduces arachidonate-derived eicosanoids such as prostaglandin E2, prostaglandin F2alpha, leukotriene-B4, and leukotriene-C4 has been explained by at least three theories. First, it is theorized that CLA displaces arachidonate in phospholipids. In cultured keratinocytes, CLA reduced incorporation of ^{14}C-arachidonate (67). In addition, dietary CLA displaced the arachidonate precursor, linoleate, in a dose-responsive manner in livers of mice fed various doses of CLA (0–1.5%) in some studies (9, 73) but not others [(2, 64); M.S. Belury,

unpublished data]. Importantly, only one study has shown that dietary CLA, rumenic acid, or t10c12-CLA reduces phospholipid-associated arachidonate in liver (49). In contrast, the remaining studies have not found that phospholipid-associated arachidonate levels are altered significantly after feeding CLA.

A second explanation for the reduction of arachidonate-derived eicosanoids by CLA is through inhibition of the constitutive enzyme, cyclooxygenase-1, and/or the inducible form, cyclooxygenase-2, at the level of mRNA, protein, or activity. An in vitro activity assay showed that CLA or individual isomers inhibited the rate of oxygenation of arachidonate in the presence of cyclooxygenase-1 (18). However, whether CLA reduces the expression of cyclooxygenase (either the constitutive form, cyclooxygenase-1, or the inducible cyclooxygenase-2) is yet to be determined.

A third theory raises the possibility that CLA or elongated and desaturated products from CLA (e.g., conjugated arachidonate) may act as substrates or antagonists for cyclooxygenase, thereby reducing available enzyme for arachidonate. It seems unlikely that conjugated-eicosatetraenoate (20:4; c5c8c11t13) can act as a substrate for cyclooxygenase because cyclooxygenase requires the 1,4 methylene interruption be farther from the carboxyl end for efficient electron abstraction by prostaglandin synthase. More likely, CLA may act antagonistically to inhibit the activity of cylooxygenase. The antagonistic property of CLA in vivo may also be regulated by the formation and accumulation of the arachidonate analogue of CLA, conjugated-eicosatetraenaote (24:4) in phospholipids. The ability of downstream metabolites of CLA to interfere with cyclooxygenase activity and/or eicosanoid production deserves further attention.

Conjugated Linoleic Acid Modulates Lipid Metabolism and Gene Expression

Until recently, the influence of CLA on lipid metabolism and gene expression in the liver and extrahepatic tissues was largely unknown. Dietary CLA alters the levels of other (nonconjugated) fatty acids in phospholipids and neutral lipids in the liver. Trends that have been observed include alterations of oleic and palmitoleic acids: In hepatic neutral lipids palmitoleic and oleic acids decrease in mice (85) and rats (3, 64, 73, 100). However, the ability of CLA to lower monounsaturated fatty acids seems to be somewhat specific for the strain and/or species of animal: We found that the monounsaturate-lowering effects of dietary CLA were more pronounced in male ZDF rats than lean (nondiabetic) littermates (M.A. Belury, unpublished data). In addition, in SENCAR mice a significant and dose-dependent increase of hepatic levels of oleic acid was observed in mice fed increasing doses of CLA (0.5–1.5%) for 6 weeks (8). The differential effects of CLA to lower monounsaturated fatty acid levels in the liver may be due to differences in isomeric compositions or dosages of CLA oils in the diet, duration of feeding, sources of non-CLA dietary fat, and/or species-, strain-, and/or metabolic status of animals (e.g., lean, diabetic).

It is possible that the altered levels of monounsaturated fatty acids such as palmitoleate and oleate may result from displacement of monounsaturated fatty acids by CLA, because CLA appears to be incorporated into similar lipid fractions as oleic acid in some studies (3, 9). In addition, CLA may alter enzymatic pathways responsible for altering fatty acid composition of lipid fractions. In fact, CLA has been shown to reduce the Δ9 desaturase index in mouse liver (61) and in cultured preadipocytes (21). In 3T3-LI preadipocytes it appears that t10c12-CLA is the most potent isomer for reducing stearoyl-CoA desaturase activity (85). The reduction of monounsaturated products of Δ9 desaturase (also called stearoyl-CoA desaturase) was at the level of reduced mRNA for stearoyl-CoA desaturase-1 regulation in liver (61). In contrast, t10c12-CLA reduction of stearoyl-CoA desaturase in cultured hepatic (HepG2) cells was not found to be associated with reduced stearoyl-CoA desaturase mRNA (22). Instead, it was proposed that CLA reduced the activity of stearoyl-CoA desaturase in cultured hepatic HepG2 cells by posttranslational modification of the protein. The finding that CLA regulates the activity of the enzyme, stearoyl-CoA desaturase, at multiple levels dependent, in part, on cell type suggests that CLA regulation of lipid metabolism and gene expression occurs through multiple signaling pathways.

CLA is readily metabolized by Δ6 desaturase to form numerous downstream products, but less is known about how CLA modulates metabolism of nonconjugated fatty acids via enzymatic systems such as Δ6 desaturase-elongase-Δ5 desaturase (Figure 3). The ability of CLA to alter levels of linoleate (18:2) and its desaturated and elongated product, arachidonate (20:4), has been observed in neutral lipid fractions of several tissues. Dietary CLA reduces arachidonate levels of mammary tissue (2), liver (8), and inguinal fat pads (88). In contrast, other studies have shown that CLA may have a modest enhancing effect on levels of neutral lipid-associated arachidonate in liver of rats (3) or epidermis of mice (56). Yet other studies show no effect of CLA on arachidonate levels of neutral lipids in fat pads (100), bone (63), liver (73), or small intestine (87). It appears that the ability of CLA to alter arachidonate levels is tissue and perhaps species dependent. Furthermore, the relevance of altered arachidonate levels in neutral lipids to modulate lipid metabolism and eicosanoid formation is not clear at the present time.

The role of CLA in modulating hepatic lipid metabolism is associated with modulating fatty acid composition. In addition, CLA appears to induce lipid accumulation of the liver in studies in which animals are fed longer than 6 weeks (8, 108) but not in shorter-term studies (2 weeks or less) (92). In fact, lipid accumulation occurs in a dose-dependent manner in female SENCAR mice fed diets with CLA (0.5–1.5%) for 6 weeks (8). Furthermore, we found that CLA-induced lipid accumulation is related to alterations in the expression of genes known to modulate lipid metabolism (9). In particular, we propose that CLA modulates lipid metabolism, in part, by a mechanism dependent on the activation of the group of nuclear transcription factors, peroxisome proliferator-activated receptors (PPARs) (Figure 4). In the liver, PPARα is a critical transcription factor

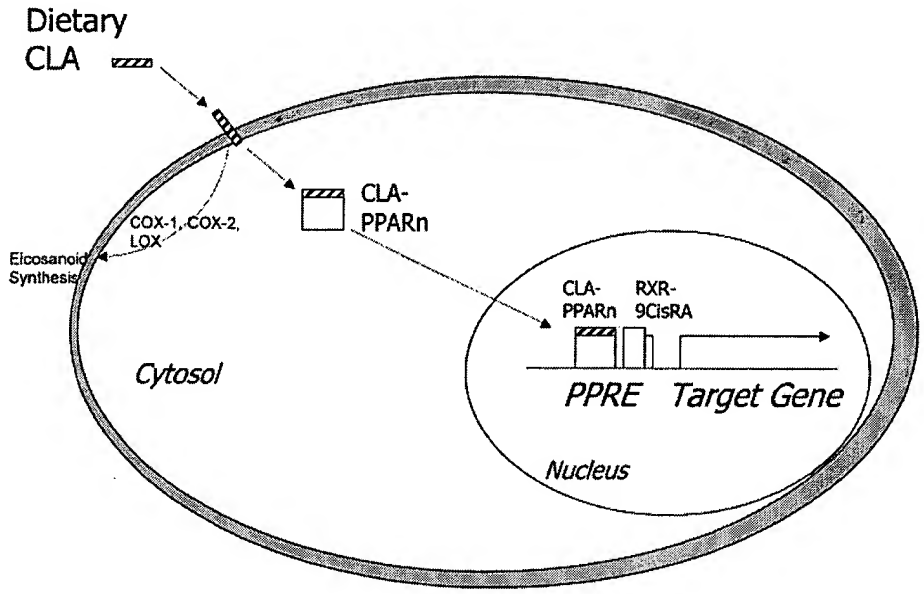

Figure 4 Schematic diagram of putative cellular and molecular mechanisms of CLA in modulating systemic conditions such as carcinogenesis, adiposity, diabetes, and cardiovascular disease.

for lipid metabolism, because several genes coding for enzymes involved with β-oxidation (either in peroxisomes or mitochondria) contain a functional peroxisome proliferator–responsive element in their enhancer regions (e.g., acyl-CoA oxidase, liver fatty acid–binding protein, cytochromep 4504A, hepatic lipoprotein lipase, and others) (95). In fact, several isomers of CLA are high-affinity ligands and activators of PPARα (74).

Because synthetic ligands for PPARα (also known as peroxisome proliferators) act as nongenotoxic carcinogens in rodent liver (89), we determined the ability of CLA to induce peroxisome proliferation and mediate apoptosis and cell proliferation in diethylnitrosamine-induced hepatic tumor promotion in rat liver. When fed to male or female Sprague-Dawley rats for up to 6 weeks, dietary CLA (0.5–1.5%) had no effects on peroxisome proliferation (73). In contrast, the prototypical peroxisome proliferator, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (Wy-14,643) induced significantly more peroxisomal area in livers (as indicated by increases in both peroxisome size and number). In this same study both dietary CLA and WY-14,643 induced PPAR-responsive genes. In F344 rats initiated with diethylnitrosamine dietary CLA was associated with increased cell proliferation and apoptosis in precancerous foci areas of rat liver (68). In contrast, Wy-14,643 induced cell proliferation without inducing apoptosis. The data suggest that CLA does not act as a typical synthetic ligand for PPARα to support hepatic

tumor promotion in rats. However, the effects of CLA on hepatic tumor promotion is yet to be determined.

Whereas evidence from our laboratory suggests that PPARα plays a role in the ability of CLA to modulate lipid metabolism, recent data using the PPARα-null mouse suggest that PPARα may not be a pivotal transcription factor for the adipose-tissue lowering effect of CLA (86). In these studies, diets with (0.5%) or without CLA were fed to PPARα null mice or wild-type for 4 weeks. Compared with wild-type (expressing PPARα), PPARα null mice (not expressing PPARα but expressing PPARβ/PPARδ and PPARγ) fed a diet with CLA had similar responses: Dietary CLA reduced adipose tissue and induced some PPAR-responsive genes in liver. It is possible that CLA modulates lipid metabolism via PPAR-independent mechanisms and/or mechanisms involving other isoforms of PPAR such as PPARβ/PPARδ and PPARγ.

The PPARγ isoform is found in extrahepatic tissues such as adipose, prostate, colon, mammary gland, and others. It has been well established that PPARγ2 is a required transcription factor in adipose tissue differentiation (77). In addition, it is known that thiazolidinediones are high-affinity ligands for PPARγ. It is therefore likely that thiazolidinediones exert their antidiabetic actions via activation of this receptor (62). In terms of affinity for PPARγ, isomers of CLA have moderate affinity for binding to and activating PPARγ (10). However, dietary CLA appears to modulate transcription of genes responsive to PPARγ in adipose tissue (41, 91) in vivo. Our initial attempts to elucidate the ability of CLA to activate PPARγ have focused on downstream metabolites of Δ6 desaturase metabolism of c9t11-CLA or t10c12-CLA. We utilized approaches to block desaturase activity to determine whether reducing metabolites will alter activation of PPARγ (10). CV-1 cells transiently transfected with murine PPARγ, luciferase–peroxisome proliferator–responsive element reporter and β-galactosidase were treated with c9t11-CLA or t10c12-CLA and the activation of PPARγ was measured. Blocking Δ6 desaturase with the synthetic inhibitor SC-26196 (78) significantly reduced the ability of CLA isomers to activate PPARγ ($P < 0.05$). These data indirectly suggest that activation of PPARγ by CLA is increased by the formation of the Δ6-desaturated products from CLA, c6c9t11-CLA, or c6t10c12-CLA. However, the activation of PPARγ by these products is yet to be measured directly.

In addition to evidence showing that CLA may induce PPARγ-responsive genes in vivo, CLA may induce the level of PPARγ itself (33). Because PPARγ2 is thought to be one of several transcription factors required for adipose tissue differentiation (77) and new evidence suggests that activators of PPARγ are protective against cancers arising in the mammary gland, colon, and prostate [reviewed in (102)], it is possible that some of the molecular mechanisms of action of CLA on obesity, diabetes, and carcinogenesis are mediated by PPARγ. Perhaps the ability of PPARγ to mediate effects of CLA occurs through increased levels of PPARγ protein (21) and/or through activation of PPARγ by downstream metabolites of CLA (e.g., desaturase and elongase products) (10).

SUMMARY: POTENTIAL ROLE OF DIETARY CONJUGATED LINOLEIC ACID IN HUMAN HEALTH

Some show that CLA-rich dairy products are associated with reduced breast cancer risk, whereas others show either no effects or even enhanced risk [reviewed in (58)]. It is estimated that the level of CLA consumed by a healthy population in the northwestern region of the United States is ~150 mg/day for women and ~200 mg/day for men (90). Based on food duplication data, most of the CLA consumed was rumenic acid (c9t11-CLA). In Canada, estimates from 7-day diet records indicated levels of intake of rumenic acid to be lower, ~94.9 + 40.6 mg/day (32). Importantly, among the 22 free-living subjects there was a wide range of intake (15–174 mg/day), and this range may be even higher in breastfeeding women in the United States (49–659 mg/day; 82). In the future it may be possible that the levels in the food supply will be amplified by feed and other biotechnological strategies. Because such small amounts of CLA (0.5% of diet) have been shown to alter the expression of genes and impact conditions such as carcinogenesis, obesity, diabetes, and atherosclerosis in experimental animals, it is possible that small amounts consumed over a prolonged period of time may exert similar beneficial effects in human beings.

Thus far, studies using supplements in humans have shown that supplementation with CLA for short periods of time (up to 12 weeks), reduces body weight and body fat in some studies (15, 101, 105). However, at least one study has found that supplementation with CLA results in elevated levels of the lipid peroxidation product, 15-keto-dihydro-prostaglandin-$F_{2\alpha}$, in urine (5). Understanding the role of CLA in modulating events associated with macronutrient metabolism suggests that CLA may be a healthy dietary component with the potential for impacting human health in the areas of cancer, obesity, diabetes, and cardiovascular disease. However, more work is needed to fully elucidate the safety and efficacy of isomers and doses that are required for exerting this breadth of potential beneficial effects. It is hoped that with improved understanding of the doses and isomers required, improvements in recommendations may be made to people regarding the intakes of CLA to improve health.

One last note concerns the role of CLA in the health of subpopulations (e.g., children, the elderly, and women during pregnancy and lactation.) During lactation the content of CLA in human breast milk is sensitive to the consumption of foods rich in CLA (35). Specifically, women who are breastfeeding and consuming a rumenic acid–rich diet produce milk with significantly higher levels of rumenic acid (13.5 μmol/g) than when consuming a low–rumenic acid diet (8.2 μmol/g) (82). In experimental animals it was recently shown that pregnant rats consuming the synthetic mixture of dietary CLA (containing both rumenic acid and t10c12-CLA at a level of 0.5%) birthed pups that had significantly longer tail lengths (as a measure of skeletal growth), heavier gastrocnemius and soleus muscles, and similar adipose mass but smaller adipocyte size (88). These data suggest that CLA in the diet may be beneficial for some parameters of growth, especially through in

utero or early availability in breast milk. However, the safety and efficacy of CLA for growing animals and subgroups in the population who have special physiological needs requires further attention.

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Dietary Conjugated Linoleic Acid Normalizes Impaired Glucose Tolerance in the Zucker Diabetic Fatty *fa/fa* Rat

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Conjugated linoleic acid (CLA) is a naturally occurring fatty acid which has anti-carcinogenic and anti-atherogenic properties. CLA activates PPARα in liver, and shares functional similarities to ligands of PPARγ, the thiazolidinediones, which are potent insulin sensitizers. We provide the first evidence that CLA is able to normalize impaired glucose tolerance and improve hyperinsulinemia in the pre-diabetic ZDF rat. Additionally, dietary CLA increased steady state levels of aP2 mRNA in adipose tissue of fatty ZDF rats compared to controls, consistent with activation of PPARγ. The insulin sensitizing effects of CLA are due, at least in part, to activation of PPARγ since increasing levels of CLA induced a dose-dependent transactivation of PPARγ in CV-1 cells cotransfected with PPARγ and PPRE X 3-luciferase reporter construct. CLA effects on glucose tolerance and glucose homeostasis indicate that dietary CLA may prove to be an important therapy for the prevention and treatment of NIDDM. © 1998 Academic Press

The dietary fatty acid, conjugated linoleic acid (CLA), has received considerable attention due to its protective properties against cancer and heart disease (reviewed in 1). CLA refers to a group of positional and geometric isomers that are derived from linoleic acid and is found in foods such as ruminant meats, pasteurized dairy products and processed cheeses (2).

Recently, we have shown that dietary CLA is associated with elevated levels of a number of genes that are responsive, in part, to the peroxisome proliferator-activated receptor-α (PPARα; 3), in rodent liver. Activation of PPARs and in particular PPARγ by thiazolidinediones has been linked to decreased circulating glucose levels and improved insulin action in animals and humans (4). The reversal of insulin resistance by thiazolidinediones appears to occur despite a lack of effect on insulin production (5,6). Since CLA may share a common mechanism of action with thiazolidinediones (i.e. via activation of PPARγ) activators, we propose CLA to be a beneficial agent in the amelioration or prevention of NIDDM.

RESEARCH DESIGN AND METHODS

Materials. Diet components were obtained from Dyets, Inc. (Bethlehem, PA). The CLA oil (Pharmanutrients Inc., Lake Bluff, IL) was ~90% CLA with the following isomeric distribution: 42% c9,t11- and t9,c11-CLA, 43.5% t10,c12-CLA, 1% c9,c11-CLA, 1% c10,c12-CLA, 1.5% t9,t11- and t10,t12-CLA, 0.5% linoleate, 5.5% oleate and 5% unidentified compound. For transient transfection experiments, CLA (98.0% purity) was purchased from NuCheck Prep, Inc (Elysian, MN). The thiazolidinedione, troglitazone (TZD, Rezulin, Parke-Davis, Ann Arbor, MI), was used as a positive control in these studies.

Animals. Male Zucker diabetic fatty (*fa/fa;* ZDF/GMI) rats and lean littermates were obtained at six weeks of age from Genetic Models, Inc. (Indianapolis, IN). Because the primary aim of the study was to determine the ability of CLA to improve insulin action, all rats were determined normoglycemic prior to assignment to experimental treatments.

After maintaining rats on experimental diets for 14 days (8 wk of age), rats were euthanized by CO_2 and cervical dislocation, blood was collected and immediately analyzed for post-prandial glucose concentrations (see below) or placed into heparinized test tubes for plasma analyses as described below. Epididymal fat pads were harvested, weighed, and immediately frozen and stored at −80°C until mRNA analyses were performed.

Experimental diets. Three isocaloric, experimental diets were formulated according to a modified AIN-76 mixture containing 6.5% (by weight) fat (7). The same amount of corn oil (5%) was used in all diets since corn oil is rich in linoleic acid, the parent compound of CLA. Research grade, tocopherol stripped lard was used to balance non-CLA diets for total fatty acid content. The diets contained either 5% corn oil + 1.5% lard + no CLA (Diet CON), 5% corn oil + 1.5% CLA (Diet CLA), or 5% corn oil + 1.5% lard + 0.2% troglitazone (Diet TZD). A dose of 1.5% CLA was chosen based on previous studies in our laboratory showing this dose modulates PPAR-associated gene expression in the liver (3) and inhibits tumorigenesis in murine skin (8). The dose of troglitazone (0.2%) used in this study has been shown to be effective at normalizing impaired glucose tolerance after 15 days (9) and suppressing elevated glucose, triglycerides, free fatty acids and urinary protein in Zucker ZDF fatty rats after 13 weeks (10).

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Glucose tolerance tests. In order to compare the effects of CLA and TZD on insulin action, a glucose tolerance test was conducted on day 11 of dietary intervention. Animals were fasted overnight (16 hr). Conscious rats were injected intraperitoneally with D-glucose (1 g/kg body weight) and blood samples were collected via the tail vein prior to the injection (time 0) and at 2, 5, 10, 15, 20, 40, 60, 120 and 180 min following injection.

Determination of blood metabolite and hormone concentrations. Blood glucose levels were determined using a One Touch glucose meter (Lifescan, Inc., Milpitas, CA). Plasma insulin concentrations were determined using commercially available radioimmunoassay kits (Linco Research, St. Charles, MO). Plasma nonesterified fatty acids were quantified using a colorimentric kit (NEFA C, Wako Chemicals, Richmond, VA).

Quantitative reverse transcription-polymerase chain reaction. The method of quantitative reverse transcriptase-polymerase chain reaction (rt-PCR) was used to measure mRNA levels of aP2 and β-actin in adipose tissue. Total RNA was isolated from adipose tissue using guanidinium thiocyanate after removing lipid with chloroform (11). Extracts of RNA were frozen (−80°C) until rt-PCR analyses were performed. Quantitative rt-PCR using recombinant (rc)RNA as an internal standard was performed as described (12). Primer sequences used for quantitation of β-actin were as described (3); primer sequences were designed for amplification of aP2 (forward: 5′ ACTGTGGCCTGAGCGACTTCTATG; reverse: 5′ AGGGGGCTT-CTGGCAAACAAT) which will yield a product of 190 basepairs.

CLA transactivation of PPARγ. African green monkey kidney cells (CV-1, ATCC# CCL-70) were grown in Eagle minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, incubated at 37°C and 5% CO_2 atmosphere. For each transfected plate (35 mm diameter), 8×10^5 cells and 625 ng psG5-PPARγ full-length expression vector (gift of J. Tugwood) was used along with 250 ng of psV-GL2-PPRE-Luciferase reporter plasmid and 250 ng of pSV-β-Galactosidase internal control plasmid. Cells were transfected using LipofectAMINE (Gibco, Life Technologies, Grand Island, N.Y.) and phenol red-free, serum reduced medium (OptiMEM I, Gibco Life Technologies, Grand Island, N.Y.). Seven hours posttransfection, charcoal stripped serum (Cocalico Biologicals, Inc. Reamstown, PA) was added to the media (10% final concentration) for an overnight incubation (16 h). Transfected cells were treated for six hours with increasing concentrations (0, 5, 10, 50, 100, 150 or 200 μM) of CLA mixture [41.2% 9(Z), 11(E)- and 9(E),11(Z)-CLA, 44.1% 10(E),12(Z)-CLA, 1.1% 9(Z),11(Z)-, 9.4% 10(Z),12(Z)-, 1.3% 9(E),11(E)-, and 10(E),12(E)-CLA, 0.7% linoleate and 2.2% unidentified compound] or troglitazone (100μM) in DMSO vehicle. Luciferase and β-galactosidase activities were assayed on cell lysates following the manufaturer's protocols (Promega, Madison, WI)

Statistical analysis. Data from these studies were analyzed by ANOVA (General Linear Model, LSD) using Statistical Analysis System (SAS; Cary, NC) or StatView for the Macintosh (Abacus Concepts, Berkley,CA).

RESULTS

Body weights and food intake. Initial body weights of rats (156.2 ± 12.1 g for lean rats, 207.0 ± 16.0 g for fatty rats) were not significantly different among diet groups. The final body weights of fatty rats fed the CLA diet were similar to CON fatty rats but were significantly lower ($p< 0.05$; data not shown) than fatty rats fed TZD. There was no overall effect of treatment on food intake in fatty rats. Daily intake of CLA and TZD in fatty rats averaged 17.12 mg/g body weight for CLA and 2.5 mg/g body weight for TZD.

In vivo glucose homeostasis. To determine if consumption of CLA could alter glucose homeostasis and insulin action, we determined the effects of dietary treatment on blood glucose and insulin concentrations and glucose tolerance. Plasma fatty acid concentrations, which play an important role in whole-body insulin action, were also quantified. At the onset of the study, fed glucose concentrations did not differ between lean and obese rats (**Figure 1, Panel A**). By d 14, fatty rats fed diet CON were markedly hyperglycemic compared to lean rats or fatty rats fed either CLA or TZD diets.

Fatty rats fed the control diet were markedly hyperinsulinemic representing a 17 fold elevation compared to lean littermate controls ($p<0.0001$; Figure 1, Panel B). Both CLA and TZD caused significant reductions in plasma insulin concentrations of fatty rats ($p<0.001$); however, CLA-fed fatty rats were still hyperinsulinemic (10.5 fold elevation) compared to lean controls. TZD was more potent than CLA in reducing plasma insulin concentrations in fatty rats; concentrations in TZD fatty rats were near to those observed in lean rats.

CLA and TZD treatments significantly lowered circulating concentrations of free fatty acids in fatty rats compared to lean littermate controls and fatty rats fed the control diet ($p<0.05$, Figure 1, Panel C).

Glucose tolerance was significantly impaired in fatty rats fed CON compared to lean controls ($p<0.01$; **Figure 2**). Treatment with either CLA or TZD for 14 d caused a reduction in the rise in glucose levels following intraperitoneal glucose injection compared to control fatty rats, and glucose levels returned to baseline levels more rapidly resulting in a lower area under the glucose curve for CLA and TZD fatty rats compared to fatty rats fed the CON diet ($p<0.01$).

PPARγ transactivation. The ability of increasing concentrations of CLA to induce PPARγ activation is illustrated in **Figure 3** (Panel A). CLA induced the PPARγ reporter gene in a dose-dependent manner, with maximal induction occuring at 150 μM CLA (3.6-fold induction).

aP2 mRNA abundance. The ability of CLA and TZD to stimulate fat cell differentiation was assessed by quantifying expression of aP2 mRNA in adipose tissue (Figure 3, Panel B). Both CLA and TZD treatment of fatty for 14 days resulted in a significant increase in aP2 mRNA abundance compared to fatty rats fed the control diet (p0.05). There was a modest, but not statistically significant, decrease in β-actin expression in the CLA- and TZD-treated groups compared to control.

DISCUSSION

We provide the first evidence that dietary CLA acts as an insulin sensitizing agent; normalizing glucose tolerance, improving hyperinsulinemia and lowering

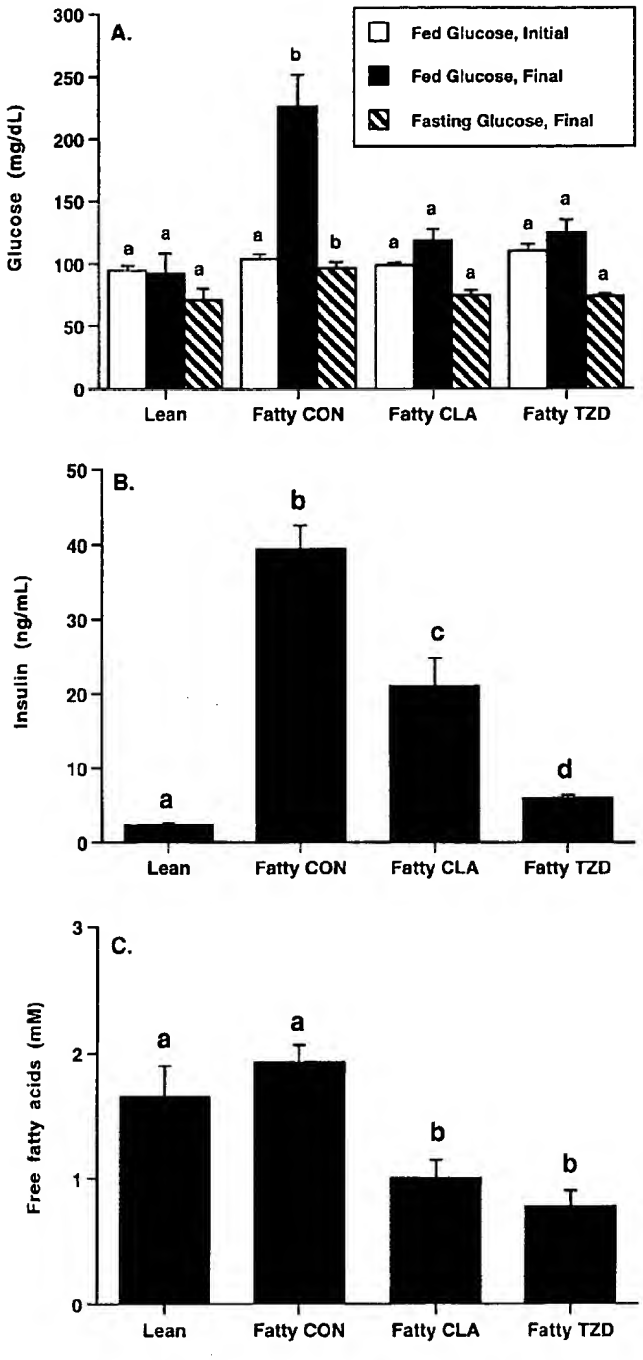

FIG. 1. Effect of dietary CLA and TZD on blood glucose (Panel A), plasma insulin (Panel B) and plasma free fatty acid (Panel C) concentrations in fed ZDF rats. Glucose concentrations in whole blood were measured in fed rats prior to the beginning of the study (initial, fed) and after rats were fed diets for 14 days (final, fed). On day 12 of the study, rats were fasted overnight (14 hr) prior to determination of fasting glucose concentrations (final, fasted). Plasma insulin and free fatty acid concentrations were determined following 14 days of treatment as described in Research Design and Methods. Values represent mean ± SEM. (n= 4 lean rats or 8 fatty rats per treatment). Lean= lean ZDF littermates fed the control diet; fatty CON= fatty ZDF rats fed the control diet; fatty CLA= fatty ZDF rats fed the conjugated linoleic-supplemented diet; fatty TZD= fatty ZDF rats fed the troglitazone-supplemented diet. [a-d]Values with different superscripts are significantly different ($p<0.05$).

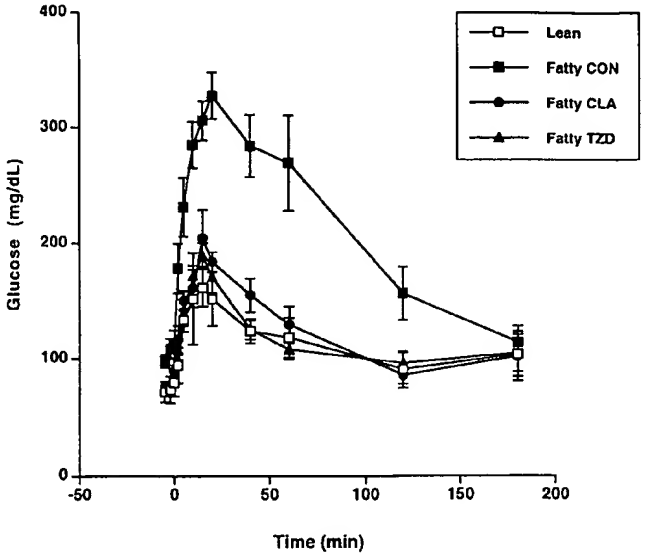

FIG. 2. Effect of dietary CLA and TZD on glucose tolerance test in ZDF lean and fatty rats. Rats were fed experimental diets for 14 days and glucose tolerance measured as described in Research Design and Methods. Values represent mean glucose (mg/dL) ± SEM. (n= 4 lean rats or 8 fatty rats per treatment). Lean= lean ZDF littermates fed the control diet; fatty CON= fatty ZDF rats fed the control diet; fatty CLA= fatty ZDF rats fed the conjugated linoleic-supplemented diet; fatty TZD= fatty ZDF rats fed the troglitazone-supplemented diet.

circulating free fatty acids, thus preventing or delaying the onset of hyperglycemia in the ZDF rat model. The striking antidiabetic properties of CLA appear to be linked to CLA-activation of PPARγ.

The ability of CLA to prevent the development of hyperglycemia in the young ZDF rat is strikingly similar to effects previously reported for the thiazolidinedione, troglitazone (13). Thiazolidinediones are thought to elicit many of their effects on adipose tissue via activation of PPARγ. Thiazolidinediones and endogenous PPAR ligands have varying degrees of affinity and activation potentials for PPARα and PPARγ (4,14,15). We have shown that dietary CLA supports significantly elevated levels of mRNA for a number of PPARα responsive genes in mouse liver (3). Therefore, we hypothesized that CLA would have insulin sensitizing effects via activation of PPARs, specifically PPARγ, in a manner comparable to the thiazolidinedione, TZD. Our findings indicate that CLA and TZD are equipotent in improving glucose tolerance and maintaining normoglycemia in pre-diabetic ZDF fatty rats, suggesting that CLA shares some or all of the insulin-sensitizing mechanisms of troglitazone, a potent and specific ligand for PPARγ.

Adipose cells are a major target tissue for PPARγ ligands, presumably due to the high level of expression of the $\gamma 2$ isoform in adipose tissue (16-18). Activation of PPARγ is a pivotal event in the adipocyte differentia-

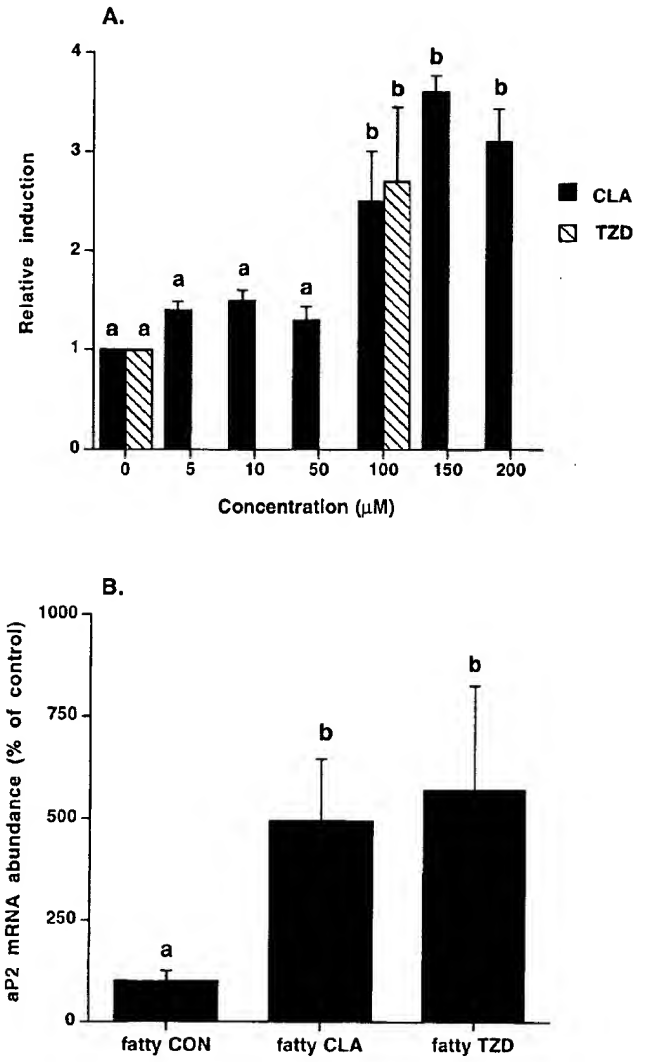

FIG. 3. Effect of dietary CLA and TZD on PPARγ activation and steady state levels of ap2 mRNA in epididymal adipose tissue from ZDF fatty rats. *Panel A:* CV-1 cells were transiently transfected with expression plasmids for psG5-human PPARγ, psV-GL2-PPRE-luciferase (reporter) and pSV-β-galacotosidase. Cells were treated for six hours with various concentrations of CLA (5 - 200 μM) or TZD (100 μM) or DMSO vehicle. Cell extracts were assayed for luciferase or β-galactosidase activities. Data represent fold induction of luciferase/β-galactosidase over DMSO vehicle value (=1. 0). *Panel B:* Rats were fed experimental diets for 14 days and aP2 mRNA abundance in epididymal fat was measured as described in Research Design and Methods. Values represent ratio of aP2/β-actin as mean % of CON values ± SEM. (n=8 fatty rats per treatment). Fatty CON= fatty ZDF rats fed the control diet; fatty CLA= fatty ZDF rats fed the conjugated linoleic-supplemented diet; fatty TZD= fatty ZDF rats fed the troglitazone-supplemented diet. $^{a\text{-}b}$Values with different superscripts are significantly different ($p<0.05$).

tion program, and is important in the transcriptional regulation of many adipocyte genes including aP2 and leptin (17,19-21). We report that both CLA and TZD significantly increased the steady state level of aP2 mRNA in adipose tissue from fatty rats, consistent with stimulation of adipocyte differentiation. More direct and compelling evidence that CLA is acting via PPARγ is the fact that CLA is able to activate PPARγ *in vitro* (Figure 3). Additionally, dietary CLA caused a slight but non-significant reduction in mean adipocyte size (K. Houseknecht, unpublished data). Thiazolidinediones such as TZD profoundly reduce adipocyte size in rodents (9,22) consistent with an increase in the number of small adipocytes due to increased differentiation. Given the increase in aP2 expression in CLA-treated fatty rats and the ability of CLA to induce PPARγ, we hypothesize that with longer term treatment or a higher dosage of CLA, we may see a proliferation of small adipocytes as observed with TZD treatment.

An important role of TZD in diabetic therapy is to reduce circulating triglycerides and free fatty acids in obese and diabetic rodents and humans (4). Inappropriately high lipid levels result in multiple pathological outcomes including inhibition of glucose utilization by skeletal muscle as proposed by Randle (23), and increased hepatic glucose output. Additionally, nonesterified fatty acids stimulate insulin secretion with varying potencies (24) and may be the signal for compensatory hyperinsulinemia in insulin resistant states (25). We observed that dietary CLA shares this potent lipid-lowering effect with TZD, since CLA was able to significantly reduce plasma free fatty acid concentrations in fatty rats. The mechanism by which CLA reduces plasma lipid concentrations may be via activation of hepatic PPARα. Fibrate hypolipidemic drugs such as gemfibrozil and clofibrate reduce plasma triglycerides presumably by activating PPARα in liver, thereby increasing fatty acid oxidation (reviewed in 26). Consistent with being a peroxisome proliferator in rodent liver, CLA induced acyl-CoA oxidase expression (a marker of peroxisomal β-oxidation) in both lean and ZDF rats and is also a PPARα activator in reporter assays (Vanden Heuvel, unpublished data). Therefore, in addition to its effects on PPARγ-related events such as adipocyte differentiation, CLA treatment may improve or prevent NIDDM symptoms in ZDF rats via a PPARα-mediated effect in liver.

In conclusion, we report that dietary CLA, like TZD, normalizes glucose tolerance and prevents the progression to hyperglycemia and diabetes in the young, prediabetic ZDF rat. Additionally, we are the first to report that at least a portion of CLA's effects are mediated at the level of the adipocyte, via the apparent activation of PPARγ and stimulation of adipocyte aP2 expression. Dietary CLA, like TZD has profound glucose-, insulin-, and free fatty acid-lowering properties which may be mediated by PPARγ and other members of the PPAR family. These data suggest that the dietary fatty acid, CLA, may prove to be an important therapy for the treatment and prevention of NIDDM.

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REDUCTION IN THE INCIDENCE OF TYPE 2 DIABETES WITH LIFESTYLE INTERVENTION OR METFORMIN

DIABETES PREVENTION PROGRAM RESEARCH GROUP*

ABSTRACT

Background Type 2 diabetes affects approximately 8 percent of adults in the United States. Some risk factors — elevated plasma glucose concentrations in the fasting state and after an oral glucose load, overweight, and a sedentary lifestyle — are potentially reversible. We hypothesized that modifying these factors with a lifestyle-intervention program or the administration of metformin would prevent or delay the development of diabetes.

Methods We randomly assigned 3234 nondiabetic persons with elevated fasting and post-load plasma glucose concentrations to placebo, metformin (850 mg twice daily), or a lifestyle-modification program with the goals of at least a 7 percent weight loss and at least 150 minutes of physical activity per week. The mean age of the participants was 51 years, and the mean body-mass index (the weight in kilograms divided by the square of the height in meters) was 34.0; 68 percent were women, and 45 percent were members of minority groups.

Results The average follow-up was 2.8 years. The incidence of diabetes was 11.0, 7.8, and 4.8 cases per 100 person-years in the placebo, metformin, and lifestyle groups, respectively. The lifestyle intervention reduced the incidence by 58 percent (95 percent confidence interval, 48 to 66 percent) and metformin by 31 percent (95 percent confidence interval, 17 to 43 percent), as compared with placebo; the lifestyle intervention was significantly more effective than metformin. To prevent one case of diabetes during a period of three years, 6.9 persons would have to participate in the lifestyle-intervention program, and 13.9 would have to receive metformin.

Conclusions Lifestyle changes and treatment with metformin both reduced the incidence of diabetes in persons at high risk. The lifestyle intervention was more effective than metformin. (N Engl J Med 2002; 346:393-403.)

TYPE 2 diabetes mellitus, formerly called non-insulin-dependent diabetes mellitus, is a serious, costly disease affecting approximately 8 percent of adults in the United States.[1] Treatment prevents some of its devastating complications[2,3] but does not usually restore normoglycemia or eliminate all the adverse consequences. The diagnosis is often delayed until complications are present.[4] Since current methods of treating diabetes remain inadequate, prevention is preferable. The hypothesis that type 2 diabetes is preventable[5,6] is supported by observational studies and two clinical trials of diet, exercise, or both in persons at high risk for the disease[7,8] but not by studies of drugs used to treat diabetes.[5]

The validity of generalizing the results of previous prevention studies is uncertain.[9] Interventions that work in some societies may not work in others, because social, economic, and cultural forces influence diet and exercise. This is a special concern in the United States, where there is great regional and ethnic diversity in lifestyle patterns and where diabetes is especially frequent in certain racial and ethnic groups, including American Indians, Hispanics, African Americans, Asians, and Pacific Islanders.[10]

The Diabetes Prevention Program Research Group conducted a large, randomized clinical trial involving adults in the United States who were at high risk for the development of type 2 diabetes. The study was designed to answer the following primary questions: Does a lifestyle intervention or treatment with

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metformin, a biguanide antihyperglycemic agent, prevent or delay the onset of diabetes? Do these two interventions differ in effectiveness? Does their effectiveness differ according to age, sex, or race or ethnic group?

METHODS

We conducted a clinical trial involving persons at 27 centers who were at high risk for diabetes. The methods have been described in detail elsewhere,[6] and the protocol is available at http://www.bsc.gwu.edu/dpp. The institutional review board at each center approved the protocol, and all participants gave written informed consent.

Participants

Eligibility criteria included an age of at least 25 years, a body-mass index (the weight in kilograms divided by the square of the height in meters) of 24 or higher (22 or higher in Asians), and a plasma glucose concentration of 95 to 125 mg per deciliter (5.3 to 6.9 mmol per liter) in the fasting state (≤125 mg per deciliter in the American Indian clinics) and 140 to 199 mg per deciliter (7.8 to 11.0 mmol per liter) two hours after a 75-g oral glucose load. These concentrations are elevated but are not diagnostic of diabetes according to the 1997 criteria of the American Diabetes Association.[11] Before June 1997, the criterion for plasma glucose in the fasting state was 100 to 139 mg per deciliter (5.6 to 7.7 mmol per liter), or ≤139 mg per deciliter in the American Indian clinics. Eligible persons were excluded if they were taking medicines known to alter glucose tolerance or if they had illnesses that could seriously reduce their life expectancy or their ability to participate in the trial. Recruitment was designed to enroll approximately half the participants from racial or ethnic minority groups. A four-step screening and recruitment process was developed to identify eligible participants.[6,12,13]

Interventions

Eligible participants were randomly assigned to one of three interventions: standard lifestyle recommendations plus metformin (Glucophage) at a dose of 850 mg twice daily, standard lifestyle recommendations plus placebo twice daily, or an intensive program of lifestyle modification. The study initially included a fourth intervention, troglitazone, which was discontinued in 1998 because of the drug's potential liver toxicity.[6] The results in the troglitazone group are not reported here.

Treatment with metformin was initiated at a dose of 850 mg taken orally once a day, with placebo tablets also given once a day initially. At one month, the dose of metformin was increased to 850 mg twice daily, unless gastrointestinal symptoms warranted a longer titration period. The initiation of treatment with half a tablet was optional. Adherence to the treatment regimen was assessed quarterly on the basis of pill counts and structured interviews. The standard lifestyle recommendations for the medication groups were provided in the form of written information and in an annual 20-to-30-minute individual session that emphasized the importance of a healthy lifestyle. Participants were encouraged to follow the Food Guide Pyramid[14] and the equivalent of a National Cholesterol Education Program Step 1 diet,[15] to reduce their weight, and to increase their physical activity.

The goals for the participants assigned to the intensive lifestyle intervention were to achieve and maintain a weight reduction of at least 7 percent of initial body weight through a healthy low-calorie, low-fat diet and to engage in physical activity of moderate intensity, such as brisk walking, for at least 150 minutes per week. A 16-lesson curriculum covering diet, exercise, and behavior modification was designed to help the participants achieve these goals. The curriculum, taught by case managers on a one-to-one basis during the first 24 weeks after enrollment, was flexible, culturally sensitive, and individualized. Subsequent individual sessions (usually monthly) and group sessions with the case managers were designed to reinforce the behavioral changes.

Outcome Measures

The primary outcome was diabetes, diagnosed on the basis of an annual oral glucose-tolerance test or a semiannual fasting plasma glucose test, according to the 1997 criteria of the American Diabetes Association: a value for plasma glucose of 126 mg per deciliter (7.0 mmol per liter) or higher in the fasting state or 200 mg per deciliter (11.1 mmol per liter) or higher two hours after a 75-g oral glucose load.[11] In addition to the semiannual measurements, fasting plasma glucose was measured if symptoms suggestive of diabetes developed. The diagnosis required confirmation by a second test, usually within six weeks, according to the same criteria. If diabetes was diagnosed, the participants and their physicians were informed and glucose-tolerance tests were discontinued, but fasting plasma glucose was measured every six months, with glycosylated hemoglobin measured annually. As long as the fasting plasma glucose concentration was less than 140 mg per deciliter, participants were asked to monitor their blood glucose and to continue their assigned study treatment. If the fasting plasma glucose concentration reached or exceeded 140 mg per deciliter, the study medication was discontinued and the participant was referred to his or her physician for treatment. Measurements of glucose and glycosylated hemoglobin (HbA_{1c}) were performed centrally. All tests were performed without interrupting the assigned treatment, except that placebo or metformin was not taken on the morning of the test. The investigators and the participants were unaware of the results of these measurements and were informed only if the results exceeded the specified threshold for a change in the treatment.

Self-reported levels of leisure physical activity were assessed annually with the Modifiable Activity Questionnaire.[16] The physical-activity level was calculated as the product of the duration and frequency of each activity (in hours per week), weighted by an estimate of the metabolic equivalent of that activity (MET) and summed for all activities performed, with the result expressed as the average MET-hours per week for the previous year. Usual daily caloric intake during the previous year, including calories from fat, carbohydrate, protein, and other nutrients, was assessed at base line and at one year with the use of a modified version of the Block food-frequency questionnaire.[17]

Statistical Analysis and Early Closure

Random treatment assignments were stratified according to the clinical center. Assignments to metformin and placebo were double-blinded. The study design and analysis followed the intention-to-treat principle. Nominal (unadjusted) P values and confidence intervals are reported.

The blinded treatment phase was terminated one year early, in May 2001, on the advice of the data monitoring board, on the basis of data obtained through March 31, 2001, the closing date for this report. By then, we had obtained evidence of efficacy on the basis of 65 percent of the planned person-years of observation. To maintain a type I error level of 0.05 for significance in pairwise comparisons of the risk of diabetes between groups, with adjustment for repeated interim analyses, the group-sequential log-rank test[18] required a P value of less than 0.0159. For pairwise comparisons of other outcomes, a Bonferroni-adjusted criterion of $P < 0.0167$ was used. The study design provided 90 percent power to detect a 33 percent reduction from an incidence of 6.5 cases of diabetes per 100 person-years, with a 10 percent rate of loss to follow-up per year.

The time to the outcome was assessed with the use of life-table methods.[19] Modified product-limit curves for the cumulative incidence of diabetes were compared with the use of the log-rank test. The estimated cumulative incidence at three years and the

Greenwood estimate of the standard error were used to calculate the number of persons who would need to be treated in order to prevent one case of confirmed diabetes during a period of three years and the associated 95 percent confidence interval. Risk reduction, heterogeneity among strata, and interactions between treatment assignments and covariates were assessed by proportional-hazards regression. Fixed-effects models with the assumption of normally distributed errors[20] were used to assess differences over time in body weight and plasma glucose and glycosylated hemoglobin values among the three groups.

RESULTS

Study Cohort and Follow-up

From 1996 to 1999, we randomly assigned 3234 study participants to one of the three interventions (1082 to placebo, 1073 to metformin, and 1079 to the intensive lifestyle intervention). Base-line characteristics, including all measured risk factors for diabetes, were similar among the three study groups (Table 1).[12] The participants were followed for an average of 2.8 years (range, 1.8 to 4.6). At the close of the study, 99.6 percent of the participants were alive, of whom 92.5 percent had attended a scheduled visit within the previous five months.

Adherence to Interventions

Fifty percent of the participants in the lifestyle-intervention group had achieved the goal of weight loss of 7 percent or more by the end of the curriculum (at 24 weeks), and 38 percent had a weight loss of at least 7 percent at the time of the most recent visit; the proportion of participants who met the goal of at least 150 minutes of physical activity per week (assessed on the basis of logs kept by the participants) was 74 percent at 24 weeks and 58 percent at the most recent visit. Dietary change was assessed only at one year. Daily energy intake decreased by a mean (±SE) of 249±27 kcal in the placebo group, 296±23 kcal in the metformin group, and 450±26

TABLE 1. BASE-LINE CHARACTERISTICS OF THE STUDY PARTICIPANTS.*

CHARACTERISTIC	OVERALL (N=3234)	PLACEBO (N=1082)	METFORMIN (N=1073)	LIFESTYLE (N=1079)
Sex — no. (%)				
Male	1043 (32.3)	335 (31.0)	363 (33.8)	345 (32.0)
Female	2191 (67.7)	747 (69.0)	710 (66.2)	734 (68.0)
Race or ethnic group — no. (%)				
White	1768 (54.7)	586 (54.2)	602 (56.1)	580 (53.8)
African American	645 (19.9)	220 (20.3)	221 (20.6)	204 (18.9)
Hispanic	508 (15.7)	168 (15.5)	162 (15.1)	178 (16.5)
American Indian	171 (5.3)	59 (5.5)	52 (4.8)	60 (5.6)
Asian†	142 (4.4)	49 (4.5)	36 (3.4)	57 (5.3)
Family history of diabetes — no. (%)	2243 (69.4)	758 (70.1)	733 (68.3)	752 (69.8)‡
History of gestational diabetes — no. of women (%)	353 (16.1)	122 (16.3)	111 (15.7)‡	120 (16.3)
Age — yr	50.6±10.7	50.3±10.4	50.9±10.3	50.6±11.3
Weight — kg	94.2±20.3	94.3±20.2	94.3±19.9	94.1±20.8
Body-mass index	34.0±6.7	34.2±6.7	33.9±6.6	33.9±6.8
Waist circumference — cm	105.1±14.5	105.2±14.3	104.9±14.4	105.1±14.8
Waist-to-hip ratio	0.92±0.09	0.93±0.09	0.93±0.09	0.92±0.08
Plasma glucose — mg/dl§				
In the fasting state	106.5±8.3	106.7±8.4	106.5±8.5	106.3±8.1
Two hours after an oral glucose load	164.6±17.0	164.5±17.1	165.1±17.2	164.4±16.8
Glycosylated hemoglobin — %	5.91±0.50	5.91±0.50	5.91±0.50	5.91±0.51
Leisure physical activity — MET-hr/wk¶	16.3±25.8	17.0±29.0	16.4±25.9	15.5±22.1

*Plus–minus values are means ±SD.

†Twenty Pacific Islanders were included in this category.

‡Information was not available for one participant.

§To convert the values for glucose to millimoles per liter, multiply by 0.05551.

¶Data are based on responses to the Modifiable Activity Questionnaire.[16] MET denotes metabolic equivalent. MET-hours represent the average amount of time engaged in specified physical activities multiplied by the MET value of each activity.

kcal in the lifestyle-intervention group (P<0.001). Average fat intake, which was 34.1 percent of total calories at base line, decreased by 0.8±0.2 percent in the placebo and metformin groups and by 6.6±0.2 percent in the lifestyle-intervention group (P< 0.001). The proportion of participants who took at least 80 percent of the prescribed dose of the study medication was slightly higher in the placebo group than in the metformin group (77 percent vs. 72 percent, P<0.001). Ninety-seven percent of the participants taking placebo and 84 percent of those taking metformin were given the full dose of one tablet (850 mg in the case of metformin) twice a day; the remainder were given one tablet a day to limit side effects.

Changes in weight and leisure physical activity in all three groups and adherence to the medication regimen in the metformin and placebo groups are shown in Figure 1. Participants assigned to the lifestyle intervention had much greater weight loss and a great-

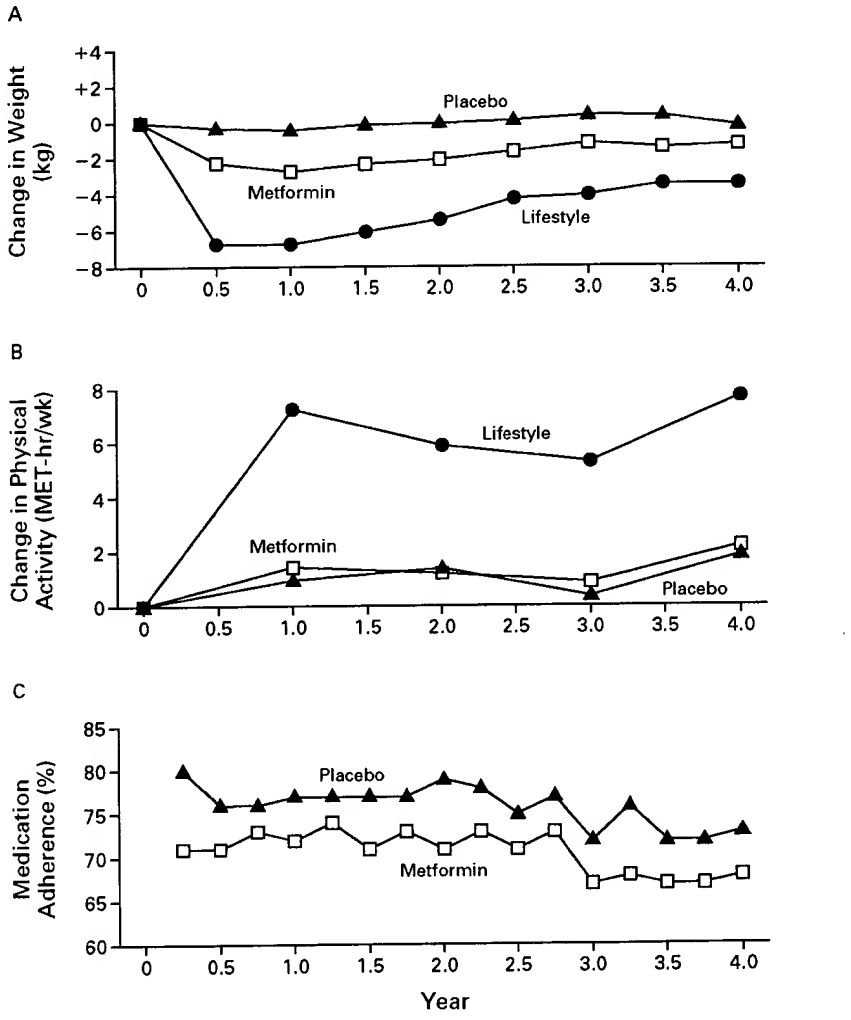

Figure 1. Changes in Body Weight (Panel A) and Leisure Physical Activity (Panel B) and Adherence to Medication Regimen (Panel C) According to Study Group.

Each data point represents the mean value for all participants examined at that time. The number of participants decreased over time because of the variable length of time that persons were in the study. For example, data on weight were available for 3085 persons at 0.5 year, 3064 at 1 year, 2887 at 2 years, and 1510 at 3 years. Changes in weight and leisure physical activity over time differed significantly among the treatment groups (P<0.001 for each comparison).

er increase in leisure physical activity than did participants assigned to receive metformin or placebo. The average weight loss was 0.1, 2.1, and 5.6 kg in the placebo, metformin, and lifestyle-intervention groups, respectively ($P<0.001$).

Incidence of Diabetes

The cumulative incidence of diabetes was lower in the metformin and lifestyle-intervention groups than in the placebo group throughout the follow-up period (Fig. 2). The crude incidence was 11.0, 7.8, and 4.8 cases per 100 person-years for the placebo, metformin, and lifestyle-intervention groups, respectively (Table 2). The incidence of diabetes was 58 percent lower (95 percent confidence interval, 48 to 66 percent) in the lifestyle-intervention group and 31 percent lower (95 percent confidence interval, 17 to 43 percent) in the metformin group than in the placebo group. The incidence of diabetes was 39 percent lower (95 percent confidence interval, 24 to 51 percent) in the lifestyle-intervention group than in the metformin group. The results of all three pairwise group comparisons were statistically significant by the group-sequential log-rank test. None of these results were materially affected by adjustment for base-line characteristics. The estimated cumulative incidence of diabetes at three years was 28.9 percent, 21.7 percent, and 14.4 percent in the placebo, metformin, and lifestyle-intervention groups, respectively. On the basis of these rates, the estimated number of persons who would need to be treated for three years to prevent one case of diabetes during this period is 6.9 (95 percent confidence interval, 5.4 to 9.5) for the lifestyle intervention and 13.9 (95 percent confidence interval, 8.7 to 33.9) for metformin.

Treatment Effects among Subgroups

Incidence rates and risk reductions within subgroups of participants and the results of tests of the homogeneity of risk reduction among subgroups are shown in Table 2; 95 percent confidence intervals for the subgroup data indicate the precision of the risk-reduction estimate for each stratum. The study had inadequate power to assess the significance of effects within the subgroups, nor were such tests planned. Significant heterogeneity indicates that treatment effects differed according to the values of the covariates. Treatment effects did not differ significantly according either to sex or to race or ethnic group (Table 2). The lifestyle intervention was highly effective in all subgroups. Its effect was significantly greater among persons with lower base-line glucose concentrations two hours after a glucose load than among those with higher base-line glucose values. The effect of metformin was less with a lower body-mass index or a lower fasting glucose concentration than with higher values for those variables. Neither interaction was explained by the other variable or by age. The advantage of the lifestyle intervention over metformin was greater in older persons and those with a lower body-mass index than in younger persons and those with a higher body-mass index.

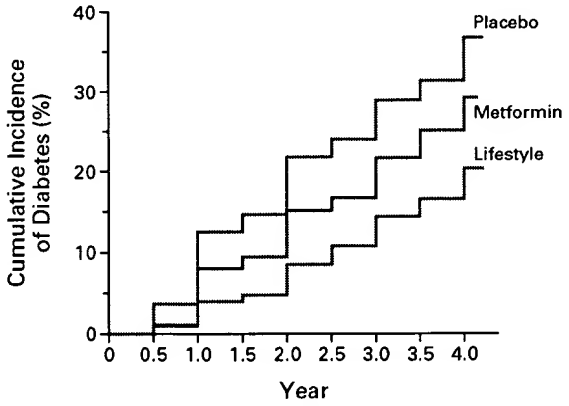

Figure 2. Cumulative Incidence of Diabetes According to Study Group.

The diagnosis of diabetes was based on the criteria of the American Diabetes Association.[11] The incidence of diabetes differed significantly among the three groups ($P<0.001$ for each comparison).

Glycemic Changes

In the first year, there was a similar reduction in the mean fasting plasma glucose values in the metformin and lifestyle-intervention groups, whereas the values rose in the placebo group (Fig. 3). The values rose in parallel in all three groups in subsequent years. There was a similar temporal pattern in the values for glycosylated hemoglobin, except that the values in the metformin group were in between those in the lifestyle-intervention and placebo groups. Figure 4 shows the percentage of participants who had normal glucose concentrations (fasting values, post-load values, and both) at each annual examination. Metformin and the lifestyle intervention were similarly effective in restoring normal fasting glucose values, but the lifestyle intervention was more effective in restoring normal post-load glucose values.

Adverse Events

The rate of gastrointestinal symptoms was highest in the metformin group, and the rate of musculoskeletal symptoms was highest in the lifestyle-intervention group (Table 3). Hospitalization and mortality rates were unrelated to treatment. No deaths were attributed to the study intervention.

TABLE 2. INCIDENCE OF DIABETES.

VARIABLE	NO. OF PARTICIPANTS (%)	INCIDENCE			REDUCTION IN INCIDENCE (95% CI)*		
		PLACEBO	METFORMIN	LIFESTYLE	LIFESTYLE VS. PLACEBO	METFORMIN VS. PLACEBO	LIFESTYLE VS. METFORMIN
		cases/100 person-yr			percent		
Overall	3234 (100)	11.0	7.8	4.8	58 (48 to 66)	31 (17 to 43)	39 (24 to 51)
Age							
25–44 yr	1000 (30.9)	11.6	6.7	6.2	48 (27 to 63)	44 (21 to 60)	8 (−36 to 37)†
45–59 yr	1586 (49.0)	10.8	7.6	4.7	59 (44 to 70)	31 (10 to 46)	41 (18 to 57)†
≥60 yr	648 (20.0)	10.8	9.6	3.1	71 (51 to 83)	11 (−33 to 41)	69 (47 to 82)†
Sex							
Male	1043 (32.3)	12.5	8.1	4.6	65 (49 to 76)	37 (14 to 54)	46 (20 to 63)
Female	2191 (67.7)	10.3	7.6	5.0	54 (40 to 64)	28 (10 to 43)	36 (16 to 51)
Race or ethnic group							
White	1768 (54.7)	10.3	7.8	5.2	51 (35 to 63)	24 (3 to 41)	36 (14 to 52)
African American	645 (19.9)	12.4	7.1	5.1	61 (37 to 76)	44 (16 to 63)	29 (−18 to 58)
Hispanic	508 (15.7)	11.7	8.4	4.2	66 (41 to 80)	31 (−9 to 56)	51 (13 to 72)
American Indian	171 (5.3)	12.9	9.7	4.7	65 (7 to 87)	25 (−72 to 68)	52 (−35 to 83)
Asian‡	142 (4.4)	12.1	7.5	3.8	71 (24 to 89)	38 (−55 to 75)	52 (−46 to 84)
Body-mass index§							
22 to <30	1045 (32.3)	9.0	8.8	3.3	65 (46 to 77)	3 (−36 to 30)†	63 (44 to 76)†
30 to <35	995 (30.8)	8.9	7.6	3.7	61 (40 to 75)	16 (−19 to 41)†	53 (28 to 70)†
≥35	1194 (36.9)	14.3	7.0	7.3	51 (34 to 63)	53 (36 to 65)†	−4 (−47 to 26)†
Plasma glucose¶							
In the fasting state							
95–109 mg/dl‖	2174 (67.2)	6.4	5.5	2.9	55 (38 to 68)	15 (−12 to 36)†	48 (27 to 63)
110–125 mg/dl**	1060 (32.8)	22.3	12.3	8.8	63 (51 to 72)	48 (33 to 60)†	30 (6 to 48)
Two hours after an oral load							
140–153 mg/dl	1049 (32.4)	7.1	4.3	1.8	76 (58 to 86)†	41 (11 to 61)	59 (27 to 77)
154–172 mg/dl	1103 (34.1)	10.3	6.6	4.4	60 (41 to 72)†	38 (13 to 56)	34 (2 to 56)
173–199 mg/dl	1082 (33.5)	16.1	12.3	8.5	50 (33 to 63)†	26 (3 to 43)	33 (9 to 51)

*CI denotes confidence interval.

†$P<0.05$ for the test of heterogeneity across strata. Age, body-mass index, and plasma glucose were analyzed as continuous variables.

‡This category includes 20 Pacific Islanders.

§The eligibility criterion was a body-mass index of at least 22 for Asians and at least 24 for all other persons.

¶To convert the values for glucose to millimoles per liter, multiply by 0.05551.

‖This category includes American Indian participants who had a fasting glucose concentration that was less than 95 mg per deciliter, according to the eligibility criteria.[6]

**This category includes 54 participants with a fasting glucose concentration of 126 to 139 mg per deciliter who were enrolled before June 1997,[6] when the eligibility criteria were changed to conform to the diagnostic criteria of the American Diabetes Association, published that year.[11]

DISCUSSION

Our results support the hypothesis that type 2 diabetes can be prevented or delayed in persons at high risk for the disease. The incidence of diabetes was reduced by 58 percent with the lifestyle intervention and by 31 percent with metformin, as compared with placebo. These effects were similar in men and women and in all racial and ethnic groups. The intensive lifestyle intervention was at least as effective in older participants as it was in younger participants. The results of our study extend previous data showing that lifestyle interventions can reduce the incidence of diabetes[7,8] and demonstrate the applicability of this finding to the ethnically and culturally diverse population of the United States. The risk reduction associated with the lifestyle intervention in our study was the same as that in a study conducted in Finland,[8] and was higher than the reductions associated with diet (31 percent), exercise (46 percent), and diet plus exercise (42 percent) in a study in China.[7]

Our lifestyle intervention was systematic and intensive, with the study participants receiving detailed, individualized counseling. The study, however, was not designed to test the relative contributions of dietary changes, increased physical activity, and weight loss to the reduction in the risk of diabetes, and the effects of these components remain to be determined.

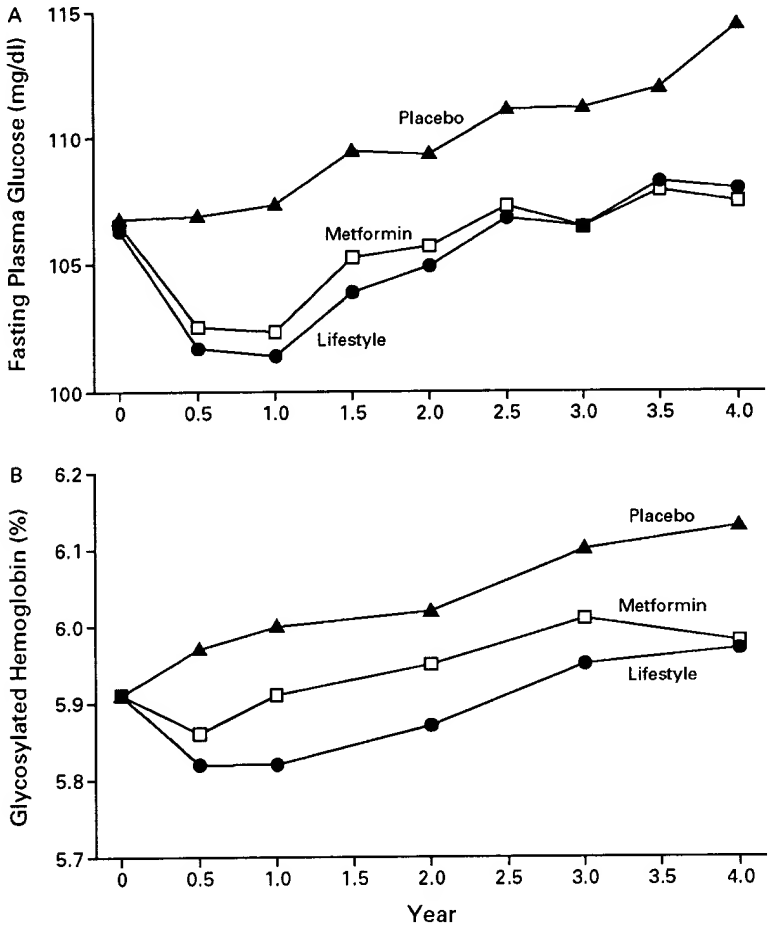

Figure 3. Fasting Plasma Glucose Concentrations (Panel A) and Glycosylated Hemoglobin Values (Panel B) According to Study Group.

The analysis included all participants, whether or not diabetes had been diagnosed. Changes in fasting glucose values over time in the three groups differed significantly ($P<0.001$). Glycosylated hemoglobin values in the three groups differed significantly from 0.5 to 3 years ($P<0.001$). To convert the values for glucose to millimoles per liter, multiply by 0.05551.

The incidence of diabetes in our placebo group (11.0 cases per 100 person-years) was higher than we had anticipated[6] and was higher than the incidence in observational studies,[21] perhaps owing to the greater frequency of glucose testing or to the selection of persons at higher risk in our study. The incidence of diabetes in the placebo group was similar among racial and ethnic groups despite differences in these subgroups in observational, population-based studies.[10] Racial and ethnic-group differences in the incidence of diabetes were presumably reduced in our study by the selection of persons who were overweight and had elevated fasting and post-load glucose concentrations — three of the strongest risk factors for diabetes.

Previous studies have not demonstrated that drugs used to treat diabetes are effective for its prevention, perhaps because of small samples and the lack of data on adherence to the prescribed regimens.[5] In contrast, metformin was effective in our study, although less so than the lifestyle intervention. Metformin was less effective in persons with a lower base-line body-

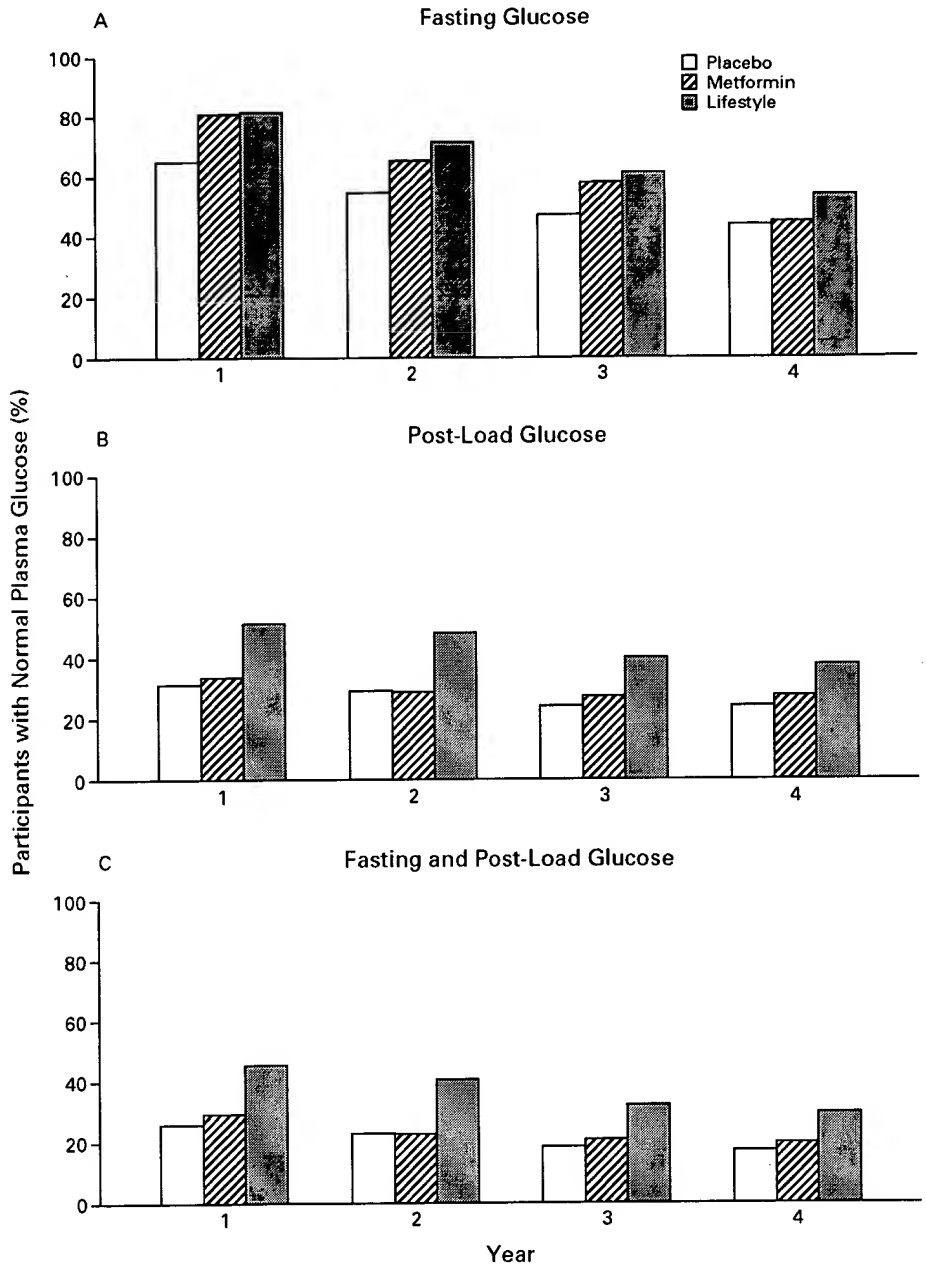

Figure 4. Participants with Normal Plasma Glucose Values, According to Study Group.

Panel A shows the proportions of participants with normal glucose values in the fasting state (<110 mg per deciliter [6.1 mmol per liter]), Panel B the proportions with normal values two hours after an oral glucose load (<140 mg per deciliter [7.8 mmol per liter]), and Panel C the proportions with normal values for both measurements. Persons in whom a diagnosis of diabetes had been made were considered to have abnormal values, regardless of the actual values at the time. By design, no participants had normal post-load glucose values at base line, but base-line fasting glucose values were normal in 67 percent of persons in the placebo group, 67 percent of those in the metformin group, and 68 percent of those in the lifestyle-intervention group. Metformin and lifestyle intervention were similarly effective in restoring normal fasting glucose concentrations, but lifestyle intervention was more effective in restoring normal post-load glucose concentrations.

TABLE 3. ADVERSE EVENTS.

EVENT	PLACEBO	METFORMIN	LIFESTYLE
Gastrointestinal symptoms (no. of events/100 person-yr)*	30.7	77.8†	12.9†
Musculoskeletal symptoms (no. of events/100 person-yr)‡	21.1	20.0	24.1†
Hospitalization			
One or more admissions (% of participants)	16.1	15.9	15.6
Rate (no. of admissions/100 person-yr)	7.9	8.4	8.0
Median stay (days)	3	3	3
Deaths (no./100 person-yr)	0.16	0.20	0.10

*Gastrointestinal symptoms included diarrhea, flatulence, nausea, and vomiting.

†$P<0.0167$ for the comparison with placebo.

‡Most participants with musculoskeletal symptoms had myalgia, arthritis, or arthralgia.

mass index or a lower fasting plasma glucose concentration than in those with higher values for these variables. The reduction in the average fasting plasma glucose concentration was similar in the lifestyle-intervention and metformin groups, but the lifestyle intervention had a greater effect than metformin on glycosylated hemoglobin, and a larger proportion of participants in the lifestyle-intervention group had normal post-load glucose values at follow-up. These findings are consistent with the observation that metformin suppresses endogenous glucose production, the main determinant of fasting plasma glucose concentrations.[22]

Rates of adverse events, hospitalization, and mortality were similar in the three groups, except that the rate of gastrointestinal symptoms was highest in the metformin group and the rate of musculoskeletal symptoms was highest in the lifestyle-intervention group. Thus, the interventions were safe in addition to being effective.

An estimated 10 million persons in the United States resemble the participants in the Diabetes Prevention Program in terms of age, body-mass index, and glucose concentrations, according to data from the third National Health and Nutrition Examination Survey.[23] If the study's interventions were implemented among these people, there would be a substantial reduction in the incidence of diabetes. Ultimately, the benefits would depend on whether glucose concentrations could be maintained at levels below those that are diagnostic of diabetes and whether the maintenance of these lower levels improved the long-term outcome. These questions should be addressed by continued follow-up of the study participants and by analysis of the main secondary outcomes — reductions in risk factors for cardiovascular disease, in the proportion of participants with atherosclerosis, and in the proportion with cardiovascular disease, which is the leading cause of death among patients with type 2 diabetes.[24,25]

Optimal approaches to identifying candidates for preventive measures remain to be determined. Although elevation of either the fasting or the post-load glucose concentration strongly predicts diabetes,[26,27] both were required for eligibility in this study. Whether the results would be similar in persons with an isolated elevation of the fasting or post-load glucose concentration or other risk factors for diabetes is likely but unknown.

In summary, our study showed that treatment with metformin and modification of lifestyle were two highly effective means of delaying or preventing type 2 diabetes. The lifestyle intervention was particularly effective, with one case of diabetes prevented per seven persons treated for three years. Thus, it should also be possible to delay or prevent the development of complications, substantially reducing the individual and public health burden of diabetes.

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Dietary fat intake and risk of type 2 diabetes in women[1-3]

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ABSTRACT

Background: The long-term relations between specific types of dietary fat and risk of type 2 diabetes remain unclear.

Objective: Our objective was to examine the relations between dietary fat intakes and the risk of type 2 diabetes.

Design: We prospectively followed 84 204 women aged 34–59 y with no diabetes, cardiovascular disease, or cancer in 1980. Detailed dietary information was assessed at baseline and updated in 1984, 1986, and 1990 by using validated questionnaires. Relative risks of type 2 diabetes were obtained from pooled logistic models adjusted for nondietary and dietary covariates.

Results: During 14 y of follow-up, 2507 incident cases of type 2 diabetes were documented. Total fat intake, compared with equivalent energy intake from carbohydrates, was not associated with risk of type 2 diabetes; for a 5% increase in total energy from fat, the relative risk (RR) was 0.98 (95% CI: 0.94, 1.02). Intakes of saturated or monounsaturated fatty acids were also not significantly associated with the risk of diabetes. However, for a 5% increase in energy from polyunsaturated fat, the RR was 0.63 (0.53, 0.76; $P < 0.0001$) and for a 2% increase in energy from *trans* fatty acids the RR was 1.39 (1.15, 1.67; $P = 0.0006$). We estimated that replacing 2% of energy from *trans* fatty acids isoenergetically with polyunsaturated fat would lead to a 40% lower risk (RR: 0.60; 95% CI: 0.48, 0.75).

Conclusions: These data suggest that total fat and saturated and monounsaturated fatty acid intakes are not associated with risk of type 2 diabetes in women, but that *trans* fatty acids increase and polyunsaturated fatty acids reduce risk. Substituting nonhydrogenated polyunsaturated fatty acids for *trans* fatty acids would likely reduce the risk of type 2 diabetes substantially. *Am J Clin Nutr* 2001;73:1019-26.

KEY WORDS Dietary fat, polyunsaturated fat, *trans* fatty acids, type 2 diabetes, risk, women

INTRODUCTION

Excess body fat resulting from an imbalance between energy intake and physical activity is the primary risk factor for type 2 diabetes (1, 2), but a role for dietary fat has also been hypothesized. However, the long-term effects of specific types of dietary fatty acids on insulin resistance and risk of type 2 diabetes remain unclear (3). Beneficial effects of diets high in monounsaturated (4, 5) and polyunsaturated (6) fatty acids relative to low-fat, high-carbohydrate diets on glucose control and insulin

See corresponding editorial on page 1001.

sensitivity have been reported, but these effects have not been seen universally (7, 8). Short-term studies documented adverse effects of *trans* fatty acid intakes on serum lipoprotein profiles (9, 10) and insulin sensitivity (11).

Epidemiologic data on dietary fats and risk of type 2 diabetes are sparse. One cross-sectional analysis reported a positive association of saturated fatty acid intake with insulin concentrations but an inverse association with polyunsaturated fatty acid intake (12). Two prospective studies that evaluated the incidence of type 2 diabetes reported no association between total dietary fat (13, 14) or specific types of fatty acids and risk of diabetes (14). However, these findings were limited by inadequate dietary assessment, a small number of endpoints, and incomplete control of confounding. In particular, these analyses did not adjust one type of fatty acid for the other, which is important because they tend to be intercorrelated (15) and may have opposing effects.

We previously reported an inverse relation of vegetable fat intake to 6-y incidence of diabetes in a large cohort of men (16), as did the Nurses' Health Study (17, 18). In the present analysis, which is based on 14 y of follow-up in the Nurses' Health Study, we examined in detail specific types of dietary fatty acids in relation to risk of type 2 diabetes. Dietary measurements were made repeatedly to reduce errors in dietary assessment and to account for changes in eating behaviors and food consumption over time. In addition, we used multivariate modeling to assess the long-term independent effects of major types of dietary fatty acids by mutually adjusting intakes of specific types of fatty acids for each other.

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TABLE 2
Relative risks (and 95% CIs) of type 2 diabetes according to quintiles of intake of specific types of dietary fat and fatty acids[1]

Variable	Quintile 1	2	3	4	5	*P* for trend
Total fat	28.9	33.9	37.2	40.6	46.1	
Age- and BMI-adjusted	1.0	0.90 (0.78, 1.02)	1.07 (0.95, 1.22)	1.07 (0.94, 1.21)	1.12 (0.99, 1.27)	0.006
Multivariate	1.0	0.87 (0.77, 1.00)	1.01 (0.88, 1.15)	0.97 (0.85, 1.10)	0.97 (0.85, 1.11)	0.96
Animal fat	17.3	21.6	25.0	29.2	36.4	
Age- and BMI-adjusted	1.0	0.94 (0.82, 1.08)	1.12 (0.98, 1.28)	1.25 (1.09, 1.42)	1.36 (1.20, 1.55)	<0.0001
Multivariate	1.0	0.90 (0.80, 1.06)	1.08 (0.93, 1.24)	1.17 (1.02, 1.35)	1.25 (1.08, 1.45)	<0.0001
Further adjustment for vegetable and *trans* fats	1.0	0.88 (0.76, 1.02)	1.00 (0.86, 1.15)	1.02 (0.88, 1.19)	0.97 (0.82, 1.15)	0.71
Vegetable fat	5.3	8.7	11.1	13.5	17.2	
Age- and BMI-adjusted	1.0	0.88 (0.79, 0.99)	0.73 (0.64, 0.82)	0.74 (0.66, 0.84)	0.72 (0.64, 0.82)	<0.0001
Multivariate	1.0	0.88 (0.78, 0.99)	0.71 (0.63, 0.81)	0.71 (0.62, 0.81)	0.68 (0.59, 0.78)	<0.0001
Further adjustment for animal and *trans* fats	1.0	0.85 (0.75, 0.96)	0.67 (0.59, 0.77)	0.65 (0.56, 0.76)	0.60 (0.51, 0.71)	<0.0001
SFA	10.7	12.8	14.3	16.0	18.8	
Age- and BMI-adjusted	1.0	1.03 (0.90, 1.18)	1.07 (0.94, 1.22)	1.21 (1.06, 1.37)	1.27 (1.12, 1.44)	<0.0001
Multivariate	1.0	1.00 (0.87, 1.15)	1.01 (0.88, 1.15)	1.10 (0.96, 1.26)	1.11 (0.97, 1.28)	0.05
Further adjustment for MUFAs, PUFAs, and *trans* fats	1.0	0.97 (0.83, 1.12)	0.96 (0.81, 1.14)	1.03 (0.86, 1.24)	0.99 (0.80, 1.21)	0.98
MUFA	10.9	13.1	14.6	16.3	19.3	
Age- and BMI-adjusted	1.0	1.08 (0.95, 1.23)	1.12 (0.98, 1.28)	1.15 (1.01, 1.31)	1.29 (1.14, 1.47)	<0.0001
Multivariate	1.0	1.05 (0.92, 1.20)	1.05 (0.92, 1.21)	1.05 (0.92, 1.21)	1.13 (0.99, 1.39)	0.07
Further adjustment for SFAs, PUFAs, and *trans* fats	1.0	1.07 (0.91, 1.25)	1.05 (0.88, 1.26)	1.02 (0.83, 1.25)	1.06 (0.84, 1.33)	0.51
PUFA	2.9	3.4	4.1	4.8	6.2	
Age- and BMI-adjusted	1.0	0.90 (0.79, 1.01)	0.83 (0.73, 0.93)	0.84 (0.75, 0.95)	0.87 (0.77, 0.99)	0.02
Multivariate	1.0	0.90 (0.80, 1.01)	0.82 (0.73, 0.93)	0.82 (0.72, 0.94)	0.85 (0.75, 0.97)	0.009
Further adjustment for SFAs, MUFAs, and *trans* fats	1.0	0.86 (0.76, 0.97)	0.77 (0.67, 0.88)	0.75 (0.65, 0.86)	0.75 (0.65, 0.88)	0.0002
trans Unsaturated fat	1.3	1.7	2.0	2.4	2.9	
Age- and BMI-adjusted	1.0	1.11 (0.97, 1.26)	1.16 (1.02, 1.32)	1.10 (0.97, 1.26)	1.26 (1.11, 1.43)	0.002
Multivariate	1.0	1.08 (0.95, 1.23)	1.11 (0.98, 1.27)	1.04 (0.91, 1.19)	1.15 (1.01, 1.32)	0.09
Further adjustment for SFAs, MUFAs, and PUFAs	1.0	1.12 (0.97, 1.29)	1.18 (1.02, 1.37)	1.14 (0.97, 1.34)	1.31 (1.10, 1.56)	0.02
Cholesterol	131	163	188	217	273	
Age- and BMI-adjusted	1.0	1.00 (0.87, 1.15)	1.12 (0.98, 1.28)	1.21 (1.06, 1.38)	1.32 (1.16, 150)	<0.0001
Multivariate	1.0	1.04 (0.90, 1.20)	1.18 (1.02, 1.37)	1.29 (1.12, 1.49)	1.42 (1.23, 1.65)	<0.0001
Further adjustment for SFAs, MUFAs, PUFAs, and *trans* fats	1.0	1.02 (0.88, 1.18)	1.16 (1.00, 1.34)	1.25 (1.08, 1.45)	1.36 (1.17, 1.59)	<0.0001

[1] Values are medians and were computed as a percentage of energy (except for cholesterol; mg/d) by quintile as the cumulative updated average. Age- and BMI-adjusted models included age (5-y categories) and BMI (11 categories). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. The multivariate models included age (5-y categories), time period (7 periods), BMI (11 categories), cigarette smoking (never, past, or current smoking of 1–14, 15–24, and ≥25 cigarettes/d), parental history of diabetes, alcohol consumption (4 categories), physical activity (metabolic equivalents/wk: 5 categories), percentage of energy from protein, and total energy intake. Dietary cholesterol was also included in models for total and specific fats.

both higher amounts of polyunsaturated fat and lower amounts of *trans* fat tended to eat less stick margarine and use more liquid vegetable oils, especially salad-dressing products.

In age- and BMI-adjusted analyses, higher total fat intake was weakly related to greater risk of diabetes (**Table 2**). In multivariate analyses controlling for known risk factors, total fat intake was no longer significantly associated with diabetes risk; adjustments for physical activity and alcohol intake largely accounted for the reduction in relative risk (RR). In age- and BMI-adjusted and multivariate analyses, vegetable fat was associated with reduced risk of diabetes. When animal and vegetable fats were both included in the same model with intake of *trans* fatty acids and known risk factors, animal fat was not associated with diabetes risk (Table 1).

Saturated and monounsaturated fatty acid intakes were each associated with an increased risk of diabetes in age- and BMI-adjusted analyses, but, in multivariate analyses including all major types of fatty acids, these associations were greatly attenuated. In contrast, polyunsaturated fatty acid intake was inversely associated with diabetes risk in all analyses.

Intakes of *trans* fatty acids was positively associated with risk of diabetes in age- and BMI-adjusted analyses. This association was slightly attenuated after adjustment for known risk factors but became stronger after other types of fat were controlled for. In addition, we examined the joint effect of polyunsaturated fatty acid and *trans* fatty acid intakes. The RR for the combination of a low *trans* fatty acid quintile and a high polyunsaturated fatty acid quintile compared with the opposite extreme was 0.66 (95%

TABLE 3
Multivariate relative risk (RR) of type 2 diabetes associated with increases in the percentage of energy from specific types of fat or fatty acids and dietary cholesterol[1]

Model	RR	95% CI	P
Model 1			
Saturated fat (5% increase in energy)	0.97	(0.86, 1.10)	0.68
Monounsaturated fat (5% increase in energy)	1.05	(0.91, 1.20)	0.52
Polyunsaturated fat (5% increase in energy)	0.63	(0.53, 0.76)	<0.0001
trans Unsaturated fat (2% increase in energy)	1.39	(1.15, 1.67)	0.0006
Cholesterol (23.9-mg/MJ increase)[2]	1.12	(1.05, 1.19)	0.0003
Model 2			
Animal fat (5% increase in energy)	0.98	(0.95, 1.02)	0.35
Vegetable fat (5% increase in energy)	0.79	(0.74, 0.84)	<0.0001
Model 3			
Total fat (5% increase in energy)	0.98	(0.94, 1.02)	0.24

[1]The multivariate models included age (5-y categories), time period (7 periods), BMI (11 categories), cigarette smoking (never, past, or current smoking of 1–14, 15–24, and ≥25 cigarettes/d), parental history of diabetes, alcohol consumption (4 categories), physical activity (metabolic equivalents/wk: 5 categories), percentage of energy from protein, and total energy intake.

[2]23.9 mg/MJ = 100 mg/1000 kcal.

CI: 0.49, 0.93; $P < 0.0001$). Dietary cholesterol was positively associated with diabetes risk in all analyses (Table 2).

Because polyunsaturated fat intake in these analyses included only linoleic acid (the primary n−6 fatty acid), we also examined the relation of marine n−3 fatty acids (eicosapentaenoic acid plus docosahexaenoic acid) to risk of diabetes. In a multivariate model that also included the major types of fat, the RRs for increasing quintiles of marine n−3 fatty acids were 1.0 (reference), 1.00 (95% CI: 0.88, 1.13), 0.93 (0.81, 1.06), 0.97 (0.84, 1.12), and 0.80 (0.67, 0.95); the P for the trend was 0.02. Because intakes of both n−6 and marine n−3 fatty acids were inversely associated with risk of diabetes, the ratio of n−6 to n−3 fatty acids was not significantly related to risk of diabetes.

To examine further the relations between different dietary fats and risk of diabetes, we also modeled the percentages of energy from specific types of fatty acids or sources of fat (animal or vegetable) as continuous variables, adjusting one type of fat for another and for known risk factors. In this model, a 5% increase in energy from vegetable fat was associated with a reduced risk of diabetes, whereas a similar increase in energy from animal fat was not associated with risk. Saturated and monounsaturated fatty acid intakes were not significantly related to diabetes risk when compared with an equivalent amount of energy from carbohydrate (**Table 3**). When included in the model with other types of fat, a 2% increase in energy from *trans* fatty acids was associated with a significantly increased risk; each increase of 23.9 mg dietary cholesterol/MJ (100 mg/1000 kcal) was associated with a 12% increased risk (Table 2).

We also estimated the effect of various isoenergetic dietary substitutions on the risk of diabetes (**Figure 1**). Replacing 5% of energy from polyunsaturated fatty acids with the same amount of energy from carbohydrates was associated with a 58% greater risk of diabetes (RR: 1.58; 95% CI: 1.31, 1.90; $P < 0.0001$). Replacing 5% of energy from saturated fatty acids with energy from polyunsaturated fatty acids was associated with a 35% lower risk (0.65; 0.54, 0.78; $P < 0.0001$). Replacing 2% of energy from *trans* fatty acids with carbohydrate was associated with a 28% lower risk (0.72; 0.60, 0.87; $P < 0.001$), but replacing *trans* fatty acids with polyunsaturated fatty acids was associated with a 40% lower risk (0.60; 0.48, 0.75; $P < 0.0001$).

We evaluated the possibility that the associations between different types of dietary fat and diabetes risk might be modified by major nondietary risk factors. The observed inverse association with polyunsaturated fatty acid intake did not appreciably differ across categories of BMI, physical activity, alcohol consumption, or family history of diabetes. However, the positive associations with *trans* fatty acids and cholesterol were observed most clearly among overweight and less physically active women (**Table 4**). In an analysis excluding women with either reported hypercholesterolemia or hypertension at baseline, the association

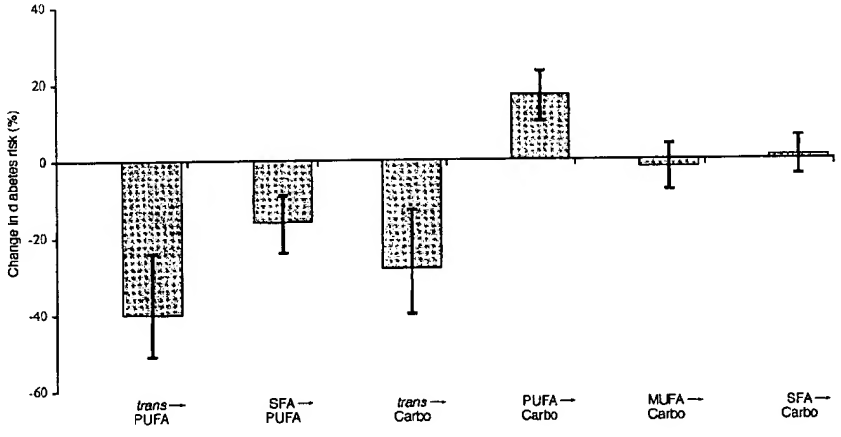

FIGURE 1. Estimated changes in risk of type 2 diabetes associated with isoenergetic substitutions of 2% of energy. Associations were adjusted for the same covariates as in Table 2. *trans*, *trans* fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; Carbo, carbohydrates; MUFA, monounsaturated fatty acids. The arrows indicate substitution of the second fat listed for the first fat listed. Bars represent 95% CIs.

TABLE 4
Relative risks (RR) of type 2 diabetes associated with increases in the percentage of energy from specific types of dietary fatty acids and dietary cholesterol according to major risk factors[1]

	Polyunsaturated fat (5% increase in energy)			*trans* Unsaturated fat (2% increase in energy)			Cholesterol (23.9-mg/MJ increase)[2]		
Risk factor	RR	95% CI	*P*	RR	95% CI	*P*	RR	95% CI	*P*
BMI (kg/m^2)									
<25 (n = 267)	0.75	(0.43, 1.31)	0.31	0.76	(0.44, 1.32)	0.33	1.04	(0.84, 1.28)	0.74
25–30 (n = 665)	0.51	(0.35, 0.74)	0.0004	1.28	(0.89, 1.86)	0.18	1.01	(0.89, 1.56)	0.91
>30 (n = 1213)	0.68	(0.52, 0.88)	0.004	1.31	(1.00, 1.72)	0.05	1.15	(1.07, 1.25)	0.0005
Physical activity level (METS/wk)									
1st and 2nd quintile (n = 1237)	0.58	(0.44, 0.76)	0.0001	1.82	(1.39, 2.38)	0.0001	1.16	(1.03, 1.26)	0.0006
3rd to 5th quintile (n = 1234)	0.69	(0.53, 0.89)	0.005	1.05	(0.88, 1.37)	0.71	1.08	(0.99, 1.18)	0.07
Alcohol consumption									
Nondrinker (n = 1054)	0.77	(0.58, 1.02)	0.07	1.11	(0.83, 1.48)	0.43	1.08	(0.98, 1.19)	0.12
0.1–5.00 mg/d (n = 634)	0.43	(0.29, 0.64)	0.0001	1.60	(1.10, 2.34)	0.01	1.15	(1.02, 1.30)	0.02
>5.0 mg/d (n = 315)	0.52	(0.31, 0.87)	0.01	1.61	(0.94, 2.74)	0.08	1.17	(1.01, 1.35)	0.04
Family history of diabetes									
Yes (n = 920)	0.62	(0.45, 0.85)	0.003	1.51	(1.10, 2.06)	0.01	1.13	(1.02, 1.24)	0.02
No (n = 1574)	0.64	(0.51, 0.81)	0.0002	1.35	(1.07, 1.70)	0.01	1.12	(1.03, 1.20)	0.006

[1] The multivariate models included age (5-y categories), time period (7 periods), BMI (11 categories), cigarette smoking (never, past, or current smoking of 1–14, 15–24, and ≥25 cigarettes/d), parental history of diabetes, alcohol consumption (4 categories), physical activity (metabolic equivalents/wk: 5 categories), percentage of energy from protein, and total energy intake. Intakes of specific types of fat and cholesterol were entered into the model simultaneously so that the effects of fats were compared with those of an equivalent amount of energy from carbohydrates. METS, metabolic equivalents.

[2] 23.9 mg/MJ = 100 mg/1000 kcal.

with intakes of polyunsaturated fatty acids (for 5% of energy: 0.64; 0.50, 0.82) and *trans* fatty acids (for 2% of energy: 1.37; 1.08, 1.74) were similar to those in the overall population.

DISCUSSION

In this large prospective study of women, we found no association between total fat intake and risk of type 2 diabetes after controlling for known risk factors. However, polyunsaturated fatty acid intake was associated with a substantial reduction in risk, and *trans* fatty acids and dietary cholesterol were associated with increased risk. We estimated that replacing 5% of energy from saturated fatty acid with energy from polyunsaturated fatty acid was associated with a 35% lower risk and that replacing 2% of energy from *trans* fatty acids with polyunsaturated fatty acid was associated with a 40% lower risk. Because the average intake of *trans* fatty acids from partially hydrogenated vegetable oils is ≈3% of energy in the United States (37, 38), our data suggest that the incidence of type 2 diabetes could be reduced by ≥40% if these oils were consumed in their original, unhydrogenated form.

Epidemiologic data on dietary fat and risk of diabetes are sparse and most of these studies are limited by incomplete control of potential confounding variables. Cross-sectional analyses have reported positive associations with saturated and monounsaturated fatty acids (12, 39) and an inverse association with polyunsaturated fatty acid intake (12). Two previous prospective studies, a 12-y follow-up study of 1462 women in Sweden (13) and a 25-y follow-up of 841 men in the Zutphen Study (14), found no significant associations between total dietary fat or specific types of fat and risk of diabetes. However, these studies were small and did not adjust simultaneously for other types of fats.

Our results regarding the lack of association with total fat and the inverse association with vegetable fat intake are also consistent with recently reported findings in a large prospective study of men (16). These findings are also consistent with earlier analyses in the Nurses' Health Study involving shorter follow-up periods (17, 18), but in the present study none of the specific types of fatty acids were significantly associated with risk of diabetes. However, our previous analyses with shorter follow-up periods did not include the mutual adjustment of one type of fatty acid for other types. Because some food sources of polyunsaturated fat, such as margarines, are also important sources of *trans* fatty acids, and because they have opposing effects, simultaneous control for the major type of fat appears to be essential to assess their independent effects. The importance of this multivariate modeling approach was previously documented for coronary heart disease (15, 40). The relative risk associated with polyunsaturated fatty acid intake adjusted for known risk factors was similar to that obtained in the age- and BMI-adjusted analysis, suggesting that confounding by lifestyle variables was only minor. However, intakes of other fats had more important confounding effects; adjustment for them strengthened the inverse association for polyunsaturated fatty acid intake and the positive association for *trans* fatty acids.

Imprecise dietary measurement and residual confounding are possible alternative explanations for some of the observed associations. However, errors in dietary assessment measures might have accounted for a lack of association but not the reverse (41). Notably, although simultaneous adjustments for other specific types of fat strengthened our findings, qualitatively similar associations were seen in analyses adjusted for age and BMI only. The repeated dietary measurements made in this study were advantageous because they allowed for fewer measurement errors and for changes in behavioral dietary patterns and food composition over time to be assessed (15, 40). On the basis of baseline dietary data from 1980 only, the associations with polyunsaturated (5% of energy) and *trans* (2% of energy) fatty acids were much weaker.

The inverse association with polyunsaturated fatty acid intake in the present analysis is consistent with the findings of a 6-y metabolic study in 102 diabetic patients that compared isoenergetic diets with different amount of linoleic acid [1.3 compared

SUBJECTS AND METHODS

Study population

The Nurses' Health Study is a longitudinal investigation of diet and lifestyle factors in relation to incidence of chronic diseases in 121 700 US female registered nurses aged 30–55 y at enrollment. The cohort was assembled in 1976 when the participants returned a mailed questionnaire about known and suspected risk factors for cancer and cardiovascular disease (19). In 1980 we assessed dietary intakes of specific types of fat and other nutrients by using a 61-item semiquantitative food-frequency questionnaire (20). In 1984 an expanded food-frequency questionnaire (116 food items) was mailed to cohort members; similar questionnaires were used to update dietary information in 1986 and 1990. For the present analysis, we used information from respondents (98 462 women aged 34–59 y) to the 1980 questionnaire. We excluded participants who did not satisfy the a priori criteria of a daily energy intake between 2092 and 14 644 kJ/d (500 and 3500 kcal/d) and those who left >10 of the 61 items on the dietary questionnaire blank. In addition, we excluded women who reported on the 1980 or a previous questionnaire a diagnosis of diabetes and those who reported cancer, myocardial infarction, angina, stroke, and coronary artery surgery because they may have modified their diet after the diagnosis. The remaining 84 204 women were followed for diabetes incidence during the subsequent 14 y (1980–1994). The follow-up rates for type 2 diabetes were 98% of the total potential person-years of follow-up. The protocol of the study was approved by the Institutional Review Board at Brigham and Women's Hospital.

Dietary assessment

We used validated semiquantitative food-frequency questionnaires to assess the participants' diets. Full descriptions of the food-frequency questionnaire in its abbreviated (61 items, 1980) and expanded (116–136 items, 1984 and on) forms, the procedures for calculating nutrient intakes, and data on reproducibility and validity in this cohort were previously reported (21–23). A common unit or portion size for each food (eg, one egg or one slice of bread) was specified and participants were asked how often on average during the previous year they had consumed that amount. The 9 responses ranged from "never or less than once per month" to "six or more times per day." Detailed information about types of fat or oil used for frying, baking, and at the table and the type of margarine usually used was collected: stick or tub in 1980 and 1984 and brand and type in 1986 and 1990. Composition values for dietary fats and other nutrients were obtained from the Harvard University Food Composition Database, derived from US Department of Agriculture sources (24) and supplemented with manufacturer's information. Food-composition data are continuously updated to account for changes in food processing and improved analytic methods. Values in 1980 for the total *trans* isomer fatty acid contents of foods were based on analyses by Enig et al (25) and Slover et al (26) and were updated by using data from the US Department of Agriculture, food manufacturers, and analyses of commonly used margarines, shortenings, and baked products at the Harvard School of Public Health (Department of Nutrition, Boston). We included all *trans* isomers of 18-carbon fatty acids. The most important determinants of *trans* fatty acids at baseline in the Nurses' Health Study were margarine; beef, pork, or lamb as a main dish; cookies (biscuits); and white bread (15). All of these food items were assessed at baseline and updated in 1984, 1986, and 1990. The polyunsaturated fat intakes reported in this study include only linoleic acid, which accounted for 81% of the total polyunsaturated fatty acid intake in our cohort. Nutrient intake was computed by multiplying the frequency of consumption of each food by the nutrient content of the specified portions, taking into account the type of fat used in preparation, including the brand, type, and year of margarine use.

Both the original and revised questionnaires provide a reasonable measure of total and specific types of fat intakes when compared with multiple 1-wk diet records; correlation coefficients for total and specific types of fat assessed by dietary records and food-frequency questionnaires ranged from 0.46 to 0.58 for the abbreviated 1980 questionnaire and from 0.48 to 0.68 for the expanded questionnaire (27). The correlation coefficient between calculated dietary intake of *trans* fatty acids and the proportion of *trans* fatty acids in adipose tissue was 0.51 (28).

Measurement of nondietary factors

In 1980 participants provided information on their weight and smoking status. We updated this information every 2 y during follow-up. The validity of self-reported weight in this cohort was previously reported (r = 0.96 between self-reported and measured weight) (29). The level of physical activity in metabolic equivalents per week was estimated based on the self-reported duration per week of various forms of exercise, with each activity weighted by its intensity level (30) according to information collected via the questionnaires at baseline, 1986, and 1992. In 1982 participants provided information on the history of diabetes in first-degree relatives.

Follow-up and ascertainment of cases

On the baseline and follow-up questionnaires that were mailed every 2 y (from 1980 to 1994), we inquired about whether diabetes had been newly diagnosed. When a diagnosis of diabetes mellitus was reported on a follow-up questionnaire, participants were asked to complete a supplementary questionnaire to confirm the report and to ascertain the date of diagnosis and details of the diagnostic tests, presenting symptoms, and medications. After women with type 1 and gestational diabetes only were excluded, the diagnosis of type 2 diabetes was established if one or more of the following criteria were met: *1*) one or more classic symptoms (excessive thirst, polyuria, weight loss, and pruritus) plus a fasting plasma glucose concentration ≥7.78 mmol/L (140 mg/dL) or a random plasma glucose concentration ≥11.11 mmol/L (200 mg/dL), 2) ≥2 elevated plasma glucose concentrations on different occasions (fasting ≥7.78 mmol/L, random ≥11.11 mmol/L, or ≥11.11 mmol/L after ≥2 h of oral-glucose-tolerance testing) in the absence of symptoms, or *3*) treatment with medication for hypoglycemia (insulin or oral hypoglycemic agents). These criteria correspond to those proposed in 1979 by the National Diabetes Data Group (31) and the World Health Organization in 1985 (32). The high validity of self-reported diabetes in this cohort on the supplementary questionnaire was previously documented and the diagnosis was confirmed by a review of medical records in 98% of cases (17). In 1997 the fasting plasma glucose concentration indicative of type 2 diabetes was lowered (≥7.0 mmol/L, or 126 mg/dL) on the basis of the American Diabetes Association's recommendation (33). In the current analyses, we used the previous criterion because at the time the follow-up was conducted, the National

TABLE 1
Baseline characteristics and risk factors for diabetes according to the intake of specific types of fat at baseline

	Polyunsaturated fat			*trans* Fat		
	Lowest quintile	Intermediate quintile	Highest quintile	Lowest quintile	Intermediate quintile	Highest quintile
Age (y)	48 ± 7[1]	46 ± 7	45 ± 7	47 ± 7	46 ± 7	46 ± 7
BMI (kg/m^2)	24 ± 4	24 ± 4	24 ± 5	24 ± 4	24 ± 4	24 ± 5
Alcohol (g/d)	10 ± 14	6 ± 10	5 ± 8	10 ± 14	6 ± 9	4 ± 7
Cholesterol (mg·MJ^{-1}·d^{-1})	51.2 ± 17.9	51.6 ± 18.4	48.3 ± 20.1	51.9 ± 24.1	50.9 ± 16.7	49.2 ± 16.7
Folate from diet (μg/d)	284 ± 122	257 ± 103	234 ± 90	313 ± 124	251 ± 96	216 ± 81
Fiber (g/d)	14 ± 6	13 ± 5	13 ± 4	16 ± 6	13 ± 4	12 ± 4
Saturated fat (% of energy)	15 ± 4	16 ± 3	15 ± 3	13 ± 4	16 ± 3	16 ± 3
Monounsaturated fat (% of energy)	14 ± 4	16 ± 4	17 ± 3	12 ± 3	16 ± 3	18 ± 3
Polyunsaturated fat (% of energy)	2 ± 0.4	4 ± 0.2	7 ± 1	3 ± 2	4 ± 1	6 ± 2
trans Fat (% of energy)	2 ± 0.5	2 ± 0.5	3 ± 0.8	1 ± 0.3	2 ± 0.1	3 ± 0.5
Family history of diabetes (%)	19	18	18	18	18	19
Current smoking (%)	32	27	28	28	28	30
Vigorous exercise ≥ 1/wk (%)	48	45	42	53	43	37
Currently receiving estrogen replacement therapy, postmenopausal women only (%)	19	19	18	19	19	17

[1] $\bar{x}$ ± SD.

Diabetes Data Group and World Health Organization definitions were the standard. Also, use of a stricter definition of type 2 diabetes can minimize false-positive results and thus enhance the validity of the observed associations (34). Deaths were identified from state vital records and the National Death Index or were reported by next of kin and the postal system; mortality follow-up was 98% complete (15).

Statistical analysis

For each participant, person-time of follow-up was counted from the date of return of the 1980 questionnaire to the date of diabetes diagnosis, to the time of return of the most recent follow-up questionnaire, or to 1 June 1994, whichever came first. Women with diabetes or cancer as indicated on a previous questionnaire were excluded from subsequent follow-up; thus, the cohort at risk included only those who remained free from diabetes or cancer at the beginning of every 2-y follow-up interval.

Women were divided into quintiles by percentage of energy from each type of fatty acid; incidence rates were calculated by dividing the number of events by person-time of follow-up in each quintile. To reduce within-subject variation and best represent the long-term diet, we used pooled logistic regression (35) to model the cumulative average of fat intake from all available dietary questionnaires up to the start of each 2-y follow-up interval in relation to diabetes incidence. During the next 2 y, for example, the fat intake from the 1980 questionnaire was related to disease incidence during the 1980–1982 and 1982–1984 time intervals and the average fat intake from the 1980 and 1984 questionnaires was related to incidence during 1984–1986. Because changes in diet after development of hypercholesterolemia, hypertension, angina, myocardial infarction, coronary artery surgery, or stroke may confound the diet-disease associations (36), we stopped updating dietary information at the beginning of the time interval during which individuals developed these endpoints.

In multivariate nutrient-density models (27), we simultaneously included energy intake, percentages of energy from protein and specific fatty acids, and other potential confounding variables. The nondietary covariates included seven 2-y time periods, age in 5-y categories, body mass index [BMI: weight (in kg) divided by the square of the height (in m) in 11 categories], smoking status (never, past, and current smoking classified into 3 categories on the basis of the number of cigarettes smoked/d: 1–14, 15–24, and ≥25), alcohol consumption (g/d in 4 categories), physical activity (metabolic equivalents/wk in 5 categories), and history of diabetes in a first-degree relative. We tested for significant monotonic trends across quintiles of fat intake by assigning each participant the median value for the category and modeling this value as a continuous variable. All P values are two-sided.

We evaluated the effects of specific types of fatty acids by expressing them as a percentage of total energy (nutrient density) and including them in models as continuous variables. When all types of fats, protein, and alcohol are included simultaneously, the coefficients from these nutrient-density models can be interpreted as the effect of exchanging energy from a specific fat for the same amount of energy from carbohydrates. We also estimated the effects of substituting one type of fat for another, using the difference between coefficients from the same model (27).

RESULTS

During 14 y of follow-up, we documented 2507 incident cases of type 2 diabetes. As previously reported (15), intakes of specific types of fat at baseline tended to correlate with one another. Intake of saturated fat correlated with intakes of monounsaturated fat (r = 0.81) and *trans* fat (r = 0.30) but not with intake of polyunsaturated fat (r = 0.01). Intake of monounsaturated fat was correlated with intakes of *trans* fat (r = 0.55) and polyunsaturated fat (r = 0.30). Intake of polyunsaturated fat was correlated with that of *trans* fat (r = 0.58). The high correlation between monounsaturated and saturated fatty acids was due to shared sources of these fats (ie, dairy products and beef). As described elsewhere (15), BMI was not appreciably associated with total or specific types of dietary fat. Women with a higher intake of *trans* fat were more likely to smoke, were less likely to engage in regular physical activity, and had lower intakes of alcohol and folate (**Table 1**). Women with a higher intake of polyunsaturated fat were less likely to smoke, less likely to engage in regular exercise, and also had lower intakes of alcohol and folate. Women who consumed

with 4.8 g/MJ, or 5.3 compared with 20 g/1000 kcal]. At the end of follow-up, there was a significant improvement in the results of oral-glucose-tolerance tests in the group that consumed the linoleic acid–enriched diet (6). A 30-wk crossover study by Heine et al (8) of 14 diabetic patients compared the long-term effects on lipoproteins of isoenergetic diets with a high ratio and those with a low ratio of polyunsaturated to saturated fatty acids (10% compared with 3% of energy intake from polyunsaturated fatty acids, respectively). The group that consumed the diet with a high ratio had an increased insulin response (assessed by in vitro binding of labeled insulin to red blood cells) and improved insulin sensitivity (indicated by a higher metabolic clearance response). However, there were no significant difference in insulin concentrations or glucose control between the 2 groups.

One proposed mechanism for the effect of polyunsaturated fatty acids on insulin sensitivity comes from observations that the fatty acid composition of cell membranes, which reflects the fatty acid composition of the diet (42), modulates insulin action; a greater saturated fatty acid content of membrane phospholipids increases insulin resistance (43). In animal models, diets enriched with polyunsaturated fatty acids enhance peripheral glucose utilization (44).

The positive association between risk of type 2 diabetes and *trans* fatty acid intake observed in our analysis is consistent with most previous studies in humans and animals, which indicate a wide variety of adverse metabolic effects on lipoprotein metabolism (10, 45) and insulin sensitivity (11, 46). In diabetic patients who consumed diets enriched with *trans* fatty acids (20% of energy) or saturated fatty acids (20% of energy) for 6 wk, the postprandial insulin response increased by 59% and 77%, respectively, compared with the effects of an isoenergetic diet with 20% of energy from nonhydrogenated monounsaturated fatty acids (11). In a preliminary report, a single meal high in *trans* fatty acids caused a reduction in insulin sensitivity (46). Although the mechanisms involved in the long-term effect of *trans* fatty acid intakes on insulin metabolism remain unclear, in vitro studies suggest a differential effect of *trans* compared with *cis* fatty acids on the regulation of insulin secretion: *trans* fatty acids potentiate glucose-stimulated insulin secretion more than do *cis*-isomers of identical chain length (47).

In a recent meta-analysis, the association between intake of *trans* fatty acids and risk of coronary heart disease in prospective studies was stronger than that predicted by the adverse effects of *trans* fatty acid intake on LDL and HDL cholesterol alone (10). The present findings suggest that this may be explained in part by disorders in carbohydrate metabolism related to higher intake of *trans* fatty acids. The positive association we observed between cholesterol intake and risk of type 2 diabetes has not been reported in other populations and requires confirmation.

In our study, the positive associations with *trans* fatty acid intakes and dietary cholesterol were observed primarily in obese and less physically active women. Although these subgroup findings need confirmation, we speculate that the effects of dietary *trans* fatty acids and cholesterol are not sufficient to cause diabetes, but in the presence of underlying insulin resistance may increase the probability of developing clinical disease. Nevertheless, the issue of multiple comparison should be considered because we looked at several dietary fatty acids simultaneously in the present analyses.

These data suggest that total fat and saturated and monounsaturated fatty acid intakes are not importantly associated with risk of type 2 diabetes in women but that dietary *trans* fatty acids increase and dietary polyunsaturated fatty acids reduce the risk. Thus, substitution of nonhydrogenated polyunsaturated fatty acids for *trans* fatty acids in the diet is likely to reduce the risk of type 2 diabetes substantially.

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Review article

Relationship of dietary fat to glucose metabolism

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Abstract

The relationship between dietary fat and glucose metabolism has been recognized for at least 60 years. In experimental animals, high fat diets result in impaired glucose tolerance. This impairment is associated with decreased basal and insulin-stimulated glucose metabolism. Impaired insulin binding and/or glucose transporters has been related to changes in the fatty acid composition of the membrane induced by dietary fat modification. In humans, high-fat diets, independent of fatty acid profile, have been reported to result in decreased insulin sensitivity. Saturated fat, relative to monounsaturated and polyunsaturated fat, appears to be more deleterious with respect to fat-induced insulin insensitivity. Some of the adverse effects induced by fat feeding can be ameliorated with omega-3 fatty acid. Epidemiological data in humans suggest that subjects with higher intakes of fat are more prone to develop disturbances in glucose metabolism, type 2 diabetes or impaired glucose tolerance, than subjects with lower intakes of fat. Inconsistencies in the data may be attributable to clustering of high intakes of dietary fat (especially animal fat) with obesity and inactivity. Metabolic studies suggest that higher-fat diets containing a higher proportion of unsaturated fat result in better measures of glucose metabolism than high-carbohydrate diet. Clearly, the area of dietary fat and glucose metabolism has yet to be fully elucidated.

Keywords: Diabetes; Diet; Fatty acids; Saturated fat; Monounsaturated fat; Polyunsaturated fat; Glucose; Insulin; Carbohydrate

1. Introduction

The amount and type of specific dietary fatty acids has a significant effect on various metabolic processes. The field studied most intensively is that of lipid metabolism, however, there is strong evidence that glucose metabolism might be affected as well. The pioneering studies about the possible effect of dietary fat on glucose metabolism in animals and humans were published by Himsworth in the 1930's [1,2]. On the basis of results garnered from a single subject fed diets containing 20 or 80% of energy as fat he concluded that "it is now securely established that the glucose tolerance of a healthy individual is determined by the composition of the diet which he is receiving" [3]. Current dietary recommendations for diabetic patients by the American Diabetes Association [4] parallel those of the National Cholesterol Education Program (NCEP) for hyperlipidemic subjects [5]. The primary emphasis is on reducing total and saturated fat, and cholesterol intakes. In this review, both animal and human studies will be discussed according to the following scheme:

2. Animal data
   2.1. Level of fat
   2.2. Type of fat (degree of saturation)
3. Human studies
   3.1. Metabolic studies/in vivo feeding trials
   3.2 Metabolic studies/in vitro studies
   3.3 Epidemiological studies

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2. Animal data

2.1. Level of fat

In an initial report on the effect of high-fat diets inducing glucose intolerance, Himsworth [6] reported that rabbits fed 50 g fresh cabbage and 120 g soybean oil per day exhibited a decreased sugar tolerance compared with rabbits fed 250 g fresh cabbage and 150 g oat bran per day. He suggested that fat feeding "retards and diminishes the action of insulin on blood sugar; prevents or delays the progressive improvement of sugar tolerance which occurs on injection of consecutive doses of glucose; and impairs the ability of insulin to diminish the hyperglycemia following intravenous injection of glucose". These differences were attributed to the availability of circulating insulin at the time of food ingestion or glucose infusion. At the time, the ability to monitor plasma insulin levels was yet to be developed. Himsworth did not report body weight in this study. It is very likely that the cabbage and oil fed rabbits were heavier at the end of the study, since they got more than twice the amount of energy compared to the cabbage and bran fed rabbits. Weight gain predisposes to disturbances in glucose metabolism. For this reason, interpretation of results of diet studies in which body weight has changed is difficult.

Little attention was paid to this observation until the late 1960's, during which a number of papers were published which confirmed Himsworth's original observation — that fat feeding in experimental animals (rats) resulted in hyperglycemia and was accompanied by impaired glucose tolerance. These observations in 'fat-adapted' rats were attributed to impaired disposal of glucose by peripheral tissues due to decreased sensitivity to endogenous insulin [7–16]. Focusing on adipose tissue, Zaragoza-Hermans and Felber [12] suggested that fat feeding induced a reduction in glucose uptake and oxidation, or conversion to fatty acids, that was secondary to decreased insulin sensitivity. However, the major site for insulin stimulated glucose utilization is muscle instead of adipose tissue. Interestingly, with the exception of Himsworth [6], the source of fat in the diets used to induce impairment of glucose metabolism ('fat-adapted') was lard (35–67% of energy). At this point no reference was made to the fatty acid composition of the diet and the consequent glucose intolerance, just to the level of fat.

Subsequent work up until 1986 continued to pursue the abnormalities in glucose metabolism induced by fat feeding, however, this work was for the most part still independent of the fatty acid composition of the diet (for the exceptions Section 2.2, 17–21). The major focus of the studies regarding the level of fat in the diet was to characterize further the type of glucose intolerance induced by fat feeding and determine the mechanism(s) for the abnormalities observed. In most of these studies the major focus was on adipose tissue.

Ip et al. [22] compared adipocytes (fat cells) isolated from Sprague-Dawley albino rats fed a high-glucose or high-fat (lard, 67% of energy) diet with respect to insulin binding. They reported that adipocytes isolated from the rats fed the high-fat diet bound less insulin and showed a decreased response to insulin (glucose oxidation) compared with rats fed the high-glucose diet. These results were consistent, regardless of whether high or low affinity binding sites were assessed. The authors concluded that fat feeding modifies the fat cell so that decreased numbers of binding sites for insulin are available. No significant changes in affinity for the hormone were demonstrable. At this point, no mention was made that a change in the fatty acid composition of the membrane, hence membrane fluidity, may have altered receptor activity.

Soon thereafter, Olefsky and Saekow [23] reported the results of a similar study. Sprague-Dawley rats were fed either a high-fat (lard, 67% of energy) or high-glucose diet. Adipocytes isolated from rats fed the high-fat diet exhibited a decrease in number of insulin receptors as well as a decrease in activity of the glucose transport system and intracellular glucose metabolism. All aspects of glucose metabolism assessed, insulin binding (a measure of receptor number), glucose transport, and intracellular capacity to oxidize glucose, were depressed. In contrast, the high-glucose fed rats exhibited decreased insulin binding but enhanced activity of both the glucose transport system and intracellular pathways of glucose metabolism compared to the high-fat fed rats. The authors stated that "It has been shown that major changes in plasma membrane phospholipid composition occur on such a diet (high-saturated fat), and these alterations in membrane structure and composition could be responsible for the changes in plasma membrane function which we have observed." This was the first time that the issue of the fatty acid composition of the high-fat diet and its subsequent effects on membrane composition and function was noted.

Lavau et al. [24] further addressed issues related to the mechanism by which high-fat diets resulted in insulin resistance in adipocytes. Wistar rats were fed a high-fat (lard, 70% of energy) or high-carbohydrate (wheat starch, 70% of energy) diet. They reported that rats consuming the high-fat diet exhibited a blunted response of glucose metabolism to insulin administration and suggested that this could be attributed to decreased intracellular capacity to utilize glucose for lipogenesis (fatty acid synthesis). Their conclusion was based on biochemical evidence indicating a decrease in the activities of intracellular enzymes associated with fatty acid synthesis.

Baxter and Schofield [25] reported that a high-fat diet (30% w/w, corn oil) fed to Wistar rats made diabetic

with streptozotocin caused a significant decrease in the extent of polydipsia (increased water intake), polyphagia (increased food intake), polyuria (increased urination), and glycosuria (glucose in the urine) relative to a high-carbohydrate diet. Thus, the authors shifted the focus of the work at this point from the effect of a high-fat diet on the induction of glucose intolerance to the effect of a high-fat diet on the control of experimentally induced diabetes. The authors stated that, "Thus, in general, the effect of the fat in the diet in the diabetic animals is to partly reverse some of the diabetes-induced enzyme changes." They attributed the beneficial effects of the high-fat diet to a decrease in the carbohydrate intake of the rats. No mention was made of why they departed from using lard (saturated fat) as the primary source of fat in the high-fat diet as had been done by previous workers in the field. It should also be noted that this is the only report suggesting a beneficial effect of fat feeding in diabetic animals. The use of an unsaturated source of dietary fat is an unlikely explanation for the discrepancy, since subsequent work, more consistent with the deleterious effects of dietary fat, also used an unsaturated source of fat [26,27]. Additionally, potentially partial preservation of β cell function after streptozotocin treatment may have contributed to the inconsistent findings of this study compared to previous work.

Salans et al. [28] focused on the effect of high-fat (50% of energy; vegetable oil) and low-fat (9% of energy) diets on glucose metabolism in adipocytes isolated from CD strain of rats. There was no effect of fat feeding on insulin binding or the cell sensitivity to insulin. However, fat feeding resulted in a significant decrease in the transport of glucose across the plasma membrane and in the maximal capacity of the cell for glucose utilization.

Hissin et al. [29] investigated the effect of fat feeding on glucose transport activity in adipocytes isolated from Sprague-Dawley rats fed a high-fat (50% of energy; vegetable oil) or low-fat diet. They reported that basal glucose transport activity was not significantly influenced by feeding the high-fat diet; however, maximally insulin-stimulated glucose transport activity decreased and was accompanied by a decrease in the glucose transport systems. Therefore, it appeared that the insulin resistance induced by consumption of the high-fat diet was attributable to a decrease in the intracellular pool of glucose transport systems. The authors suggested that this decrease may be a reflection of a general systematic impairment of insulin action. The changes observed due to fat feeding were unrelated to adipose tissue mass per se.

Shifting the tissue of interest to muscle, which is the major site of insulin action, Grundleger and Thenen [30] assessed the effect of feeding a high-fat (67% of energy, hydrogenated fat) or high-carbohydrate (67% of energy, corn starch) diet to lean Zucker rats (a strain of rat whose liter mates become spontaneously obese) on insulin binding and glucose metabolism in soleus muscle. They reported that both in vivo and in vitro measures of insulin resistance were enhanced by fat feeding. These included decreased insulin receptor number independent of a change in receptor affinity, decreased insulin-stimulated glucose transport, and pathway-specific alterations in basal and insulin-stimulated glucose metabolism.

Storlien et al. [31] assessed the effect of fat feeding on in vivo insulin resistance and energy expenditure in rats. Wistar rats were pair fed high-fat (59% of energy, safflower oil) or high-carbohydrate (one-third sucrose, two-thirds starch) diets. The investigators concluded that the high-fat diet resulted in whole-body insulin resistance resulting from resistance both at the level of individual peripheral tissues (decreased glucose utilization in skeletal muscle and brown adipose tissue) and liver. They suggested that a reduction in postprandial thermogenesis could predispose the rats to the development of obesity. Fat feeding had no effect on basal metabolic rate. In this same year the first report on type of fat and fat induced glucose intolerance was recorded (see Section 2.2).

Kraegen et al. [32] investigated peripheral insulin action in Wistar rats fed high-fat (59% of energy, safflower oil) or high-carbohydrate (one-third sucrose, two-thirds starch) diets. Fat feeding did not result in hyperinsulinemia but mildly reduced basal glucose metabolism in skeletal muscle (diaphragm) and substantially reduced glucose metabolism in other tissues, such as adipose tissue and heart, despite hyperinsulinemia. When the rats were made hyperinsulinemic, insulin resistance was exacerbated (decreased mean whole body net glucose utilization in both skeletal muscle and adipose tissue). The authors concluded that fat feeding resulted in insulin resistance primarily due to decreases in the oxidative capacity of skeletal muscle.

Chisholm and O'Dea [26] designed a study to determine the effects of a high-fat diet on glucose sensitivity in rats with experimentally induced diabetes. Sprague-Dawley rats were fed either a high-fat (66% of energy; corn oil and lard) or a low-fat (12% of energy; wholemeal flour) diet. Both diets had a polyunsaturated-to-saturated fatty acid (P/S) ratio of 1:3. Mild insulin deficiency was induced with streptozotocin in half the rats in each group. Fasting glucose concentrations, but not insulin concentrations, were higher in the rats fed the high-fat diet, independent of streptozotocin treatment. This is in contrast with the report by Baxter and Schofield [25]. The rate of removal of an intravenous glucose load was impaired in both groups of rats fed the high-fat diet, independent of streptozotocin treatment, and could not be attributed to a defect in basal or insulin-stimulated glucose utilization in peripheral

tissues. Response to exogenous insulin was similar in all groups of rats. It was concluded that the high-fat diet, in either control or diabetic rats, did not result in alterations in glucose metabolism or insulin sensitivity in muscle.

Watarai et al. [33] further investigated the mechanism responsible for the insulin resistance observed after high-fat feeding in rats. Sprague-Dawley rats were fed either a high-fat (60% of energy, lard) or high-carbohydrate (61% of energy, wheat flour) diet. Consumption of the high-fat diet resulted in insulin resistance manifested by hyperinsulinemia and decreased response of adipocytes to insulin. Decreased insulin-stimulated glucose uptake was attributed to a decrease in the insulin-stimulated phosphorylation of the β-subunit of the insulin receptor. For the most part, this study was consistent with the results of previous work [22–24,28,29]. The different measures of insulin sensitivity of adipose tissue used in the different studies to date reflected a refinement of the technology available to evaluate the insulin/glucose relationship.

Nagy et al. [34] investigated the effect of a high-fat diet on tissue specific insulin sensitivity. Weanling Sprague-Dawley rats were fed either laboratory chow or a high-fat (31% lard) diet. Fat fed rats gained significantly more body weight and had higher glucose, insulin, and triglyceride levels. The activity of the insulin receptor's β-subunit (insulin-sensitive autophosphorylation, tyrosine kinase activity toward a synthetic substrate, and content of 'activated' phosphotyrosyl-containing insulin receptors) was assessed. These measures were increased in the partially purified receptors from kidney basolateral membranes and decreased in those from the liver and skeletal muscle of the rats fed the high-fat diet. The authors concluded that 'tissue-specific alterations in transmembrane signaling induced by high-fat feeding in target tissues for insulin... might contribute to the observed insulin resistance'.

To further investigate the mechanism by which fat feeding decreases insulin sensitivity, Pedersen et al. [35] investigated the activity of two glucose transporters (GLUT1 and GLUT4) in adipocytes as a function of dietary modification. Four groups of Sprague-Dawley rats were each fed diets that differed in fat and calorie contents. This review will focus on two groups, high-fat (lard) and high-carbohydrate (mainly sucrose and glucose) fed rats. Rats fed the high-fat diet exhibited lower plasma glucose and insulin concentrations than the rats fed the high-carbohydrate diet. Basal rates of glucose transport were similar between the two groups of animals, however, maximal insulin-stimulated rates of glucose transport were lower in the high-fat group. High-fat feeding resulted in a decrease in the levels of GLUT4 protein and mRNA. Additionally, GLUT4 protein concentrations per adipocyte decreased relatively more than the glucose transport rate, suggesting up-regulation of functionally active GLUT4 transporters in the plasma membrane or enhanced glucose transporter intrinsic activity. The authors suggested that the mechanism responsible for the alterations in GLUT4 expression as a result of the high-fat diet might be related to the hypoinsulinemia, since similar responses of GLUT4 have been observed in both diabetic and fasted rats. Unexplained was the observation of lower plasma glucose concentrations in the high-fat fed rats compared to the high-carbohydrate fed rats relative to other work addressing the effect of fat feeding and insulin resistance.

Pascoe et al. [27] investigated dietary fat-induced insulin resistance by assessing the effect of fat feeding in control and mildly diabetic (very low-dose neonatal streptozotocin induced non-insulin dependent diabetes mellitus or type 2 diabetes) rats. Wistar rats consumed either a high-fat (safflower oil, 59% of energy) or high-carbohydrate (starch) diet. The fat fed rats exhibited reductions in basal glucose clearance and basal hepatic glucose output. Furthermore, whole-body and tissue-specific insulin sensitivity were significantly depressed in fat-fed rats compared to starch-fed rats.

Hedeskov et al. [36] assessed the effect of long-term fat feeding in NMRI mice. As previously observed, initially in rabbits, and subsequently in different strains of rats, fat feeding results in impaired glucose tolerance and plasma insulin concentrations increase. These observations are consistent with the development of insulin resistance and with reduced peripheral and hepatic uptake of glucose. Post-receptor metabolic disorders of the liver, muscle, and adipose tissue were identified by assessing the activities of a number of intracellular enzymes involved with glucose and fatty acid metabolism. The authors concluded that the fat-induced insulin resistance observed was similar to that demonstrated in human type 2 diabetes.

Traianedes et al. [37] assessed the effect of a high-fat diet on the metabolic control in Sprague-Dawley rats made mildly diabetic with streptozotocin. Rats were fed a low-fat (wholemeal flour) or high-fat diet (butter or lard). Fat feeding alone induced hyperglycemia, and this was exacerbated in the diabetic rats. This hyperglycemia was due predominantly to an increase in hepatic glucose production, both in the basal and hyperinsulinemic states. The authors concluded that a high-fat diet induces hepatic insulin resistance leading further to an overproduction of glucose by the liver.

Barnett et al. [38] studied the effect of the inhibition of fatty acid oxidation with etomoxir (inhibitor of carnitine palmitoyltransferase) on fat feeding in rats made diabetic with streptozotocin. In this study Sprague-Dawley rats (both diabetic and control) consumed a high-fat diet (50% of energy, butter): diabetic rats exhibited significantly higher fasting glucose and plasma triglyceride levels than normal rats. The inhibi-

tion of fatty acid oxidation reduced fasting glucose concentrations independent of alterations in fasting insulin levels. These data are consistent with improved insulin sensitivity, perhaps induced by a shift in available substrate for energy utilization; from fat to glucose.

Storlien et al. [21] assessed the effect of high-fat diets (59% of energy, safflower oil) on glucose sensitivity in Wistar rats. They suggested that the mechanism for the fat induced insulin resistance is attributable to either an increased accumulation of triglyceride in skeletal muscle, thereby providing an alternate fuel to glucose, or to a change in the fatty acid composition of the membrane, altering fluidity and perhaps receptor activity. (See Section 2.2 for additional results on type of fat).

Capito et al. [39] assessed the effect of fat induced diabetes on mouse pancreatic islet insulin secretion, insulin biosynthesis and glucose metabolism. Glucose stimulated insulin secretion was decreased in islet cells from fat fed mice and was accompanied by a significant decrease in islet glucose oxidation. Insulin secretion stimulated in islet cells by non-carbohydrate compounds was moderately, but not significantly, increased. Fat feeding resulted in decreased pancreatic insulin content and pro-insulin mRNA. These findings are similar to those found in humans diagnosed with type 2 diabetes.

Iwanishi and Kobayashi [40] used fat fed rats (Sprague-Dawley, 60% of energy saturated fat, lard) as a model of type 2 diabetes to evaluate the effect of an oral hypoglycemic agent, 5-[4-[2-(5-ethyl-12-parietal)ethoxyl]-benzyl]-2,4-thiazolidinedione (pioglitazone), as a form of therapy. Two weeks of treatment with pioglitazone resulted in a decrease in hyperlipidemia and hyperinsulinemia. These data suggested that insulin sensitivity was increased in the drug treated high-fat fed rats. The authors further reported that attenuation of the hyperinsulinemia was attributable to increased insulin-stimulated autophosphorylation of insulin receptors. Pioglitazone had no effect on insulin binding to the hindlimb skeletal muscle. The benefits of pioglitazone treatment of fat-induced glucose intolerance was due to an increased sensitivity to insulin that resulted from activation of the tyrosine kinase activity of the insulin receptors.

Storlien et al. [21,31] had initially reported that the insulin resistance observed in rats as a result of fat feeding was primarily due to increased triglyceride storage in the muscle and/or changes in membrane fatty acid composition. To pursue this work further Storlien et al. [41] assessed the effect of altering intracellular skeletal muscle triglyceride levels with benfluorex on insulin resistance. Using their standard model for diet-induced diabetes (59% of energy fat as safflower oil; Storlien et al. [31]) fat feeding had no significant effect on plasma triglyceride levels, but increased skeletal muscle triglyceride content. Treatment with benfluorex normalize stored triglyceride levels in muscle and prevented the development of skeletal muscle insulin resistance. The authors concluded that their work "supported the hypothesis that the development of muscle insulin resistance..." is linked to local or systemic oversupply of lipid". A possible mechanism for the effect on glucose utilization of the increased lipid availability in muscle tissue might be the glucose/fatty acid cycle [42,43]: with increasing availability of triglycerides in the muscle tissue more triglycerides are being utilized to produce energy for muscle tissue and less glucose is utilized as a source of energy, leading to decreased glucose utilization. However, in a recent study insulin stimulated glucose transport was reduced in muscles of fat-fed rats under anoxic conditions under which fatty acid oxidation should not have occurred [44].

Kusunoki et al. [45] assessed the effect of glucocorticoid blockade (antiglucocorticoid RU486) on fat feeding (59% of energy; safflower oil) induced insulin resistance in skeletal muscles of Wistar rats. Treating the rats with RU486 resulted in a significant improvement in the skeletal muscle insulin resistance produced by fat feeding. The authors stated that "The results suggest that glucocorticoids play, in a tissue-specific manner, a role in the maintenance and/or production of insulin resistance produced by high-fat feeding".

From a review of the literature to this point on fat feeding in experimental animals and insulin resistance it can be concluded that high-fat diets result in insulin resistance, as evidenced in rabbits, rats (multiple strains), and mice. Striking has been the rather arbitrary selection of the type of fat (saturated and/or polyunsaturated) used to induce the insulin resistance. Multiple mechanisms for fat-induced glucose intolerance have been invoked and the syndrome is likely to be multifactorial. In rats, high fat feeding has been found to decrease basal and insulin-stimulated glucose utilization [30,31]. A decrease in the binding of insulin to its receptor resulting in an impairment in the action of some enzymes, e.g. pyruvate dehydrogenase and tyrosine kinase [33,34,46] has also been reported as well as the decrease in the active form of glycogen synthase leading to a defect in glucose storage as glycogen [36]. A decrease in the amount of GLUT4 has been reported in some [47,48], but not all studies [44]. In addition, high fat diets fed to mice have been reported to diminish GLUT4 translocation to the plasma membrane [49]. It has also been suggested that fatty acids cause β-cell insensitivity to glucose by down regulating acetyl-CoA carboxylase, the enzyme that catalyzes the formation of a key regulator of fatty acid oxidation, malonyl-CoA [50]. In a β-cell line, a prolonged exposure to palmitate, oleate, and linoleate resulted in high basal insulin release and suppression of glucose-induced insulin secre-

tion. No significant difference among individual fatty acids was reported [50].

2.2. Type of fat (degree of saturation)

To this point, insulin resistance had been reported to result from high-fat diets; however, the major fat source in the diet was either high in saturated fat [22–24,33–35,37,38,40], hydrogenated fat ([30] (otherwise undefined)), or polyunsaturated fat [6,21,25,27–29,31,32,41,45]. Work related to the impact of type of fatty acids, rather than total amount of fat, on glucose homeostasis is far more limited.

Directly addressing the issue of dietary fat type on insulin resistance, van Amelsvoort et al. [17] isolated adipocytes from Wistar rats born to dams fed high-fat diets enriched in either palm (saturated) or sunflower (polyunsaturated) oil while pregnant and then raised on a similarly enriched diet. They observed that feeding palm oil resulted in a lower rate of insulin stimulated glucose uptake and insulin binding to cells (lower number of low-affinity binding sites) than feeding sunflower oil. Assessing the fatty acid composition of the adipocyte phospholipid fraction (primarily membrane lipid), they reported that the profile reflected that of the fat fed to the dams and later to the offspring. They concluded that "diet-induced differences in fatty acid composition of the phospholipid... caused a difference in the physicochemical properties of the fat cell membranes, which could be responsible for the observed differences in insulin response." The absence of a chow-fed group precluded assessing whether feeding a high-fat sunflower oil diet itself induced insulin resistance; however, from previous work [6,21,25,27–29,31,32,41,45], it can be assumed that it did.

Also focusing on the type of fat, Storlien et al. [18] investigated the effect of replacing $\approx 6\%$ of linoleic acid in a high-fat diet containing 59% of energy from safflower oil with long-chain omega-3 fatty acids derived from fish oil (tuna) for 24 days. They reported that this modest substitution prevented the development of insulin resistance at the whole-body level in Wistar rats. The major sites of action were the liver and skeletal muscle. No effect was seen in heart and lung. The authors postulated that potential mechanisms responsible for the effects seen included alterations in the production of various prostaglandins, thromboxanes, and prostacyclins (unique to long-chain omega-3 fatty acids; eicosapentaenoic and docosahexaenoic acids), changes in membrane fluidity, or inhibition of very low density lipoprotein synthesis by the liver, hence, a switch in the fuel available for energy metabolism. It is important to note that the authors state that although "...the relation of fat intake to insulin resistance in humans has been established largely on the basis of epidemiological studies... there is little evidence concerning alterations in insulin sensitivity in humans after the kind of changes in dietary fat described here".

Field et al. [19,20] fed control and diabetic (streptozotocin-induced) rats high-fat diets with either a low-P/S ratio (0.2) or high-P/S ratio (2.1) for 42 days. Consumption of the low-P/S ratio diet resulted in an decrease in the polyunsaturated fatty acid content of membrane phospholipids of adipose tissue from both the control and diabetic rats. This was accompanied by a decrease in insulin binding in the control rats but not in the diabetic rats. Feeding the diet with a low-P/S ratio, hence higher saturated fat intake, decreased the rates of insulin stimulated glucose transport, oxidation and lipogenesis.

Further pursuing the relationship between fat type and insulin resistance, Storlien et al. [21] assessed the effect of high-fat diets (59% of energy) enriched in saturated fat (136 g edible tallow and 203 g safflower oil), monounsaturated fat (olive oil), polyunsaturated fat (safflower oil), polyunsaturated fat + long-chain omega-3 fatty acids (237 g safflower oil + 102 g fish oil), polyunsaturated fat + short-chain omega-3 fatty acids (268 g safflower oil + 71 g linseed oil, *n*-3 linolenic acid, 11% of energy), and saturated fat + short-chain omega-3 fatty acids (268 g edible tallow + 71 g linseed oil) on insulin resistance. The authors reported that regardless of the fatty acid profile, high-fat diets resulted in increased insulin resistance relative to chow-fed animals. Insulin resistance was greatest in the group of rats consuming the saturated fat-enriched diet relative to the other two diets. As previously reported by these authors [18], the addition of long-chain omega-3 fatty acids, eicosapentaenoic and docosahexaenoic acids (20:5*n*-3 and 22:6*n*-3, respectively), resulted in the amelioration of insulin resistance. In contrast, addition of similar amounts of a short-chain omega-3 fatty acid, α-linolenic acid (18:3*n*-3), had no effect on insulin resistance. The addition of α-linolenic acid to the saturated fat-enriched diet resulted in levels of insulin resistance similar to those of the chow-fed animals. There was a strong relationship between insulin-stimulated glucose metabolism and the percent of long-chain omega-3 fatty acids in the phospholipid fraction of red quadriceps (skeletal muscle).

The work cited in this section is particularly important because it established that relatively short-term (30 days) feeding of high-fat diets, independent of fatty acid profile, resulted in an impairment of insulin sensitivity, that saturated fat, relative to monounsaturated and polyunsaturated fats, was more deleterious with respect to fat-induced insulin insensitivity, and that some of the effects induced by feeding a high-fat diet could be ameliorated by adding omega-3 fatty acids to the diet as reported in multiple recent studies [51,52]. Differential effects of the omega-3 fatty acids according to chain length and number of double bonds on high-fat diets

containing different fatty acid profiles were noted. Differential effects of individual fatty acids (varying in chain length) have yet to be addressed. However, this is an important area given the recent report that long chain polyunsaturated fatty acid decrease glucose-6-phosphate dehydrogenase pre-mRNA accumulation whereas monounsaturated fatty acids do not [53]. Additionally, differences among (for example) saturated fatty acids may be important since there is evidence that under Ca^{2+} free conditions, palmitate and myristate augment insulin secretion by pancreatic β-cells to same extent as glucose, whereas other saturated fatty acids do not [54].

Regarding the quantitative effects of fatty acid subgroups on glucose homeostasis, the fatty acid modifications in the above mentioned studies have been extreme, and in general, the modification of either the amount of fat or the fatty acid composition has been much more substantial than likely to occur in human diets. For this reason the extrapolation of the quantitative effect of fatty acid groups to a human diet is difficult at this time.

3. Human studies

3.1. Metabolic studies/in vivo feeding trials

Studies addressing both the level and type of fat will be reported together in this section because in many cases both were varied simultaneously. Dividing the studies into two categories would be arbitrary, and any benefits of forcing such an order are outweighed by the confusion it would create.

In 1927, Sweeney noted that the response to a dextrose tolerance test, used diagnostically for diabetes mellitus, was affected by the composition of the diet consumed a few days prior to the test [55]. Subjects (male medical students) were asked to consume diets high in protein, carbohydrate, or fat (olive oil, butter, mayonnaise made with egg yolk, and 20% cream). Glucose tolerance curves were highest after the subjects consumed the high-fat diet, intermediate after the high-protein diet, and lowest after the high-carbohydrate diet. These differences were attributed to differences in "... the activation of the insulin stimulating hormone...". That is, subjects habituated to the high-carbohydrate diet were the most primed, so to speak, for the rapid glucose influx, whereas subjects habituated to the high-fat diet were the least primed. Similar results were reported by Himsworth [1] and Conn [56].

The first study directly addressing the effect of the quality of fat on glucose metabolism was reported by Kinsell et al. [57] about 40 years ago. There was one subject in this study: a man with type 1 diabetes. He consumed a safflower oil enriched diet and a diet enriched in synthetic palmitic acid-oleic acid triglyceride. The substitution of safflower oil for the palmitic acid-oleic acid triglyceride resulted in a decrease in the need of exogenous insulin.

Early work suggesting that glucose metabolism was improved by low-fat diets was limited to normal subjects or relatively small numbers of patients with type 2 diabetes [1,58–62]. However, in general, when the effect of a low-fat diet was assessed while controlling for body weight changes, no significant effect in either diabetic control (plasma glucose levels) or insulin requirements was observed [60,63].

In 1971, Brunzell et al. [64] assessed the effect of high-fat (40% of energy, unidentified source) and fat-free formula diets on fasting and postprandial (after an oral glucose load) plasma glucose and insulin levels in normoglycemic and mildly hyperglycemic subjects. The authors reported that fasting plasma glucose and insulin levels decreased in both groups of subjects after consumption of the low-fat diet. In response to an oral glucose tolerance test, total integrated glucose area under the curve was lower after consumption of the low-fat diet. No significant change in the incremental insulin area or percent insulin area was reported as a result of altering the diet. The authors suggested that the "...improvement in glucose tolerance (in response to the low-fat diet) may have been due in part to the decreased fasting glucose levels..." and that "...one possible effect of the carbohydrate-enriched diets is to increase the sensitivity to insulin of tissue sites of insulin action". Plasma triglyceride levels increased in response to the relatively short period (10 days) of time the subjects consumed the low-fat diet. These result has been confirmed in more recent studies [65–71]. The observation of increased plasma triglyceride levels is notable in light of a report by Albrink and Davidson [72] suggesting that increased triglyceride levels caused insulin resistance. However, although plasma triglyceride levels are typically increased in subjects with insulin resistance and type 2 diabetes, there is no evidence in humans that an increase in plasma triglyceride levels itself causes insulin resistance. Although not assessed at the time, increased plasma triglyceride levels were likely accompanied by a decrease in high density lipoprotein levels. Work by Stone and Connor [63] suggested that increased plasma triglyceride levels induced by high carbohydrate diets, and presumably decreased high density lipoprotein levels, was transient.

Obesity is associated with elevated basal plasma insulin levels. Grey and Kipnis [73] assessed the effect of high- or low-fat diets fed at levels to maintain body weight or allow weight loss in obese females on basal insulin concentrations. High-fat (53% of energy, fat type unreported) or low-fat (18% of energy) diets composed of natural foods were fed to subjects for 3-week periods. Basal insulin levels were lower on the high-fat

diet. Obese subjects then consumed high-fat (74% of energy) and low-fat (1% of energy) liquid formula diets in amounts that resulted in weight loss. Basal insulin levels were lower after consuming the high-fat diet compared to the low-fat diet. Re-feeding the low-fat diet, despite weight loss, resulted in an increase in basal insulin levels. After an oral glucose load, insulin secretory response was lower after the subjects consumed the high-fat diet compared to the low-fat diet. The authors concluded that "...the hyperinsulinemia characteristic of obesity may be a result, in part, of dietary factors rather than exclusively a consequence of the insulin antagonism associated with obesity". The extreme nature of the diets limits the generalizability of the data.

Beck-Nielsen at al. [74] supplemented the diets of normal volunteers with either 1000 kcal of sucrose or cream. They reported that there was no effect on fasting plasma insulin or glucose concentrations, or insulin sensitivity, but, there was a significant decrease in specific cell-bound insulin in the fat-supplemented group.

Vessby et al. [75] assessed the effect of substituting polyunsaturated for saturated fat in the diets of hyperlipidemic subjects. The total fat content of the diet was 44% of energy, the diet high in polyunsaturated fatty acids had a P/S ratio of 2, whereas the diet high in saturated fatty acids had a P/S ratio of 0.2. The diets were isocaloric. The diet high in saturated fatty acids resulted in less favorable glucose tolerance, especially in subjects with type 4 hyperlipidemia (elevated triglyceride levels).

Collier and coworkers conducted a number of studies on the effect of acute fat feeding on blood glucose and insulin levels. They reported that fat + carbohydrate feeding lowered blood glucose and increased insulin levels compared with carbohydrate feeding alone [76,77]. In a follow-up study [78], they assessed the effect of acute high-fat (37.5 g fat as butter + 75 g carbohydrate as lentils) and very low-fat (lentils) feeding on glucose-dependent insulinotropic polypeptide (GIP). GIP potentiates glucose-induced insulin secretion. Ingesting the high-fat relative to the very low-fat diet resulted in increased plasma insulin and C-peptide levels in response to an iv. glucose administration. The increased insulin levels were attributed to increased insulin secretion (as evidenced from increased C-peptide levels). It could not be determined whether increased insulin secretion was attributable to GIP or some other factor. Gatti et al. [79] have examined the acute effect of feeding different types of fats on plasma glucose and insulin responses in ten healthy subjects. Olive and corn oils decreased the postprandial area under the glucose curve, whereas butter delayed the plasma glucose rise without changing the area under the curve. No effect on plasma insulin concentrations was reported.

Coulston et al. [65] compared two isocaloric diets, one with 40% of energy as carbohydrate, 41% of energy as fat and the other with 60% of energy as carbohydrate, 21% of energy as fat, in healthy volunteers. No difference in fasting plasma glucose or insulin concentrations were found, whereas the concentration of plasma triglycerides, and insulin and triglyceride response to a test meal was significantly higher after consumption of the high-carbohydrate diet. In a follow-up study [66], a high-fat diet (40% of energy as both fat and carbohydrate) and a high-carbohydrate diet (60% of energy as carbohydrate, 20% of energy as fat) were consumed for 15 days each by patients with type 2 diabetes. The incremental glucose and insulin responses to a normal meal cycle (08:00–16:00 h), and fasting and postprandial triglyceride levels were higher after the high-carbohydrate compared to the high-fat diet. Subsequent work demonstrated that plasma glucose and insulin concentrations were higher throughout the day when the subjects consumed the high-carbohydrate diet compared to the high-fat diet [67]. The fasting plasma triglyceride levels were increased during the high-carbohydrate diet period.

Garg et al. [68] compared a high-carbohydrate diet (60% of energy as carbohydrate, 25% of energy as fat (9% of energy as monounsaturated fat)) to a high-fat diet enriched in monounsaturated fat (50% of energy as fat (33% of energy as monounsaturated fat), 35% of energy as carbohydrate) in patients with type 2 diabetes. The high-fat diet resulted in lower mean plasma glucose concentrations and reduced insulin requirement as well as decreased plasma triglyceride concentration compared to a high-carbohydrate diet. The authors reported in a follow-up study that in type 2 diabetics similar dietary perturbations had little effect on plasma glucose and insulin responses to a standard breakfast and an euglycemic hyperinsulinemic glucose clamp despite differences in plasma triglyceride levels [69]. In a third study by this group [70], a high-carbohydrate diet (55% of energy as carbohydrate, 30% of energy as fat (10% of energy as monounsaturated fat)) was found to increase fasting plasma triglyceride concentrations and day long glucose, insulin and triglyceride concentrations compared to a high-fat diet enriched in monounsaturated fat (45% of energy as fat (25% of energy as monounsaturated fat), 40% of energy as carbohydrate).

O'Dea et al. [80] assessed the effect of a high-fat (55% of energy, $P/S = 0.26$) and low-fat (12% of energy, $P/S = 0.71$) diet in the treatment of patients with type 2 diabetes. Basal plasma glucose and insulin levels were higher after the subjects consumed the high-fat diet. Levels of glucose and insulin were also higher in the postprandial state after a glucose load. Whether the effects observed were due to level of fat, independent of a concomitant increase in the saturated fat content of the diet, was not assessed.

Abbott et al. [81] assessed the effect of replacing fat (43% of energy to 33% of energy), primarily saturated fat, with carbohydrate, in the diet of Pima Indians diagnosed as having type 2 diabetes. The diets were isocaloric. The outcomes suggested that replacing dietary saturated fat with complex carbohydrate, independent of cholesterol intake, had no adverse effect on either fasting glucose or glucose concentrations 2 h after the oral glucose load. Even in the absence of weight loss, significant improvements in the lipoprotein profile of the subjects were noted.

Heine et al. [82] varied the P/S ratio (0.3 and 1.0) of diets fed to patients with type 2 diabetes. Serum total and LDL cholesterol concentrations declined after of the diet within the high P/S ratio. In addition, modest increases in insulin-mediated glucose disposal and no differences in glycemic control or blood glucose, plasma insulin, or C peptide responses were reported. The authors concluded that patients with type 2 diabetes may benefit from a reduction in the saturated fat intake with respect to plasma lipid concentrations without an apparent effect in glycemic control or carbohydrate tolerance.

Traianedes [83] assessed the effect of dietary fats on plasma glucose and insulin levels by altering the fatty acid composition of the diet ingested 12 h prior to a standard breakfast. The supplemental fat represented 53% of total energy intake and was provided as safflower oil (polyunsaturated), olive oil (monounsaturated), butter (saturated), or medium-chain triglycerides. No effect of a single dose of any of the fats tested on plasma glucose and insulin levels added to a standard meal was reported. Similar results by this group had been reported in relation to the effect of a fat-containing breakfast on carbohydrate tolerance after lunch [84]. Fat-impaired glucose tolerance was similar, whether the ingested fat was butter (saturated) or peanut butter (unsaturated). Fukagawa et al. [85] investigated the effect of replacing fat with carbohydrate in the diet of normal subjects. There was a decline in the total saturated, monounsaturated, and polyunsaturated fat content of the diet. The results of the study suggested that reducing the fat content of the diet improved "carbohydrate economy by enhanced peripheral sensitivity to insulin".

Swinburn et al. [86] varied the fat content of the diet of normal Caucasians and Pima Indians (genetically predisposed to type 2 diabetes). The diets contained either 15% fat or 50% fat (P/S ratio from 2.7 and 0.5, respectively), the major change being a decrease in saturated fat content of the diets. Glucose-mediated glucose disposal, β-cell function, and glucose tolerance deteriorated after subjects consumed the high-fat diet. Caucasians and Pima Indians responded similarly, although the adverse effects of the high fat diet on plasma lipid levels were more pronounced in the Pima Indians.

Howard et al. [87] addressed the issue of substituting complex carbohydrate for saturated fat in the diet of Caucasians and Pima Indians in a series of two studies, one in which the total fat content of the diet was reduced from 42 to 21% of energy (saturated fat from 21 to 6%) and another in which the fat content of the diet was reduced from 50 to 15% of energy (saturated fat from 22 to 3%). There was no effect of diet on glucose tolerance after subjects were switched from the 42 to 21% fat diet; however, there was an improvement in glucose-mediated glucose disposal and insulin secretion when they were switched from the 50 to 15% fat diet. The reduction in saturated fat intake had a positive effect on the plasma lipid profile (total and LDL cholesterol) of the subjects in both studies. No effect on plasma triglyceride levels was reported. The authors suggested that "In individuals having a wide range of obesity and glucose tolerance, substitution of complex carbohydrates for saturated fat has beneficial effects on lowering low density lipoprotein cholesterol levels and possibly improving glucose tolerance and insulin secretion but without having any adverse effects on lipoprotein metabolism or energy expenditure".

Borkman et al. [88] compared the effect of high-fat (50% of energy, 24% saturated fat) and low-fat (20% of energy, 9% saturated fat) diets on glucose tolerance. Mean whole-body glucose uptake during glucose infusion, and fasting blood glucose and serum insulin concentrations were similar, regardless of the dietary fat intake. Plasma lipid profiles were more favorable after subjects consumed the low-fat diet. With respect to plasma lipid profiles, similar findings were reported by Chen et al. [89].

Bonanome et al. [90] compared a high-fat diet (40% of energy fat (25% of energy monounsaturated fat), 45% of energy carbohydrate) to a high-carbohydrate diet (60% of energy carbohydrate, 25% of energy fat (10% of energy monounsaturated fat)) in 19 patients with type 2 diabetes consumed in isocaloric amounts. No difference in glycosylated hemoglobin or fasting plasma glucose, insulin, C-peptide or triglyceride concentrations were found between the diets.

Patients with type 2 diabetes consumed both a high-fat diet (40% of energy as both fat and carbohydrate, 29% of energy as monounsaturated fat) and a high-carbohydrate diet (60% of energy as carbohydrate, 20% of energy as fat (13% of energy as monounsaturated fat)) for 15 days [71]. The high-fat diet decreased both postprandial plasma glucose and insulin concentrations, as well as fasting plasma triglyceride concentration. Insulin mediated glucose disposal measured by the euglycemic hyperinsulinemic clamp was higher in the high-fat diet compared to the high-carbohydrate diet.

In patients with type 2 diabetes, Rasmussen et al. [91] examined the effect of isocaloric diets either high in fat (50% of energy as fat (30% of energy as monounsatu-

rated fat), 30% of energy as carbohydrate) or high in carbohydrate (50% of energy as carbohydrate, 30% of energy as fat (10% of energy as monounsaturated fat)) for a period of 3 weeks each. The high-fat diet resulted in lower fasting blood glucose, lower average blood glucose and lower peak blood glucose levels during a normal meal cycle compared to a high-carbohydrate diet. Again, in male type 2 diabetic subjects, Campbell et al. [92], reported that diets high in fat and enriched in monounsaturated fat (37% of energy as fat (22% of energy as monounsaturated fat), 40% of energy as carbohydrate) relative to high-carbohydrate (55% of energy as carbohydrate, 22% of energy as fat (8% of energy as monounsaturated fat)) resulted in a lower mean glucose profile, 24-h urinary glucose excretion, and fasting plasma triglyceride concentrations.

Uusitupa et al. [93] compared the effect of 9 or 20% saturated fat diets (total dietary fat 40% of energy) on glucose metabolism in normal female subjects. After consumption of the high saturated fat diet, in response to a standard glucose tolerance test, the area under the curve was greater and glucose disappearance rate was slower than after the subjects consumed the low saturated fat diet. In a follow-up study Schwab et al. [94] also had subjects consume high-fat diets, of which either 21 or 10% was saturated fat. In contrast to the previous study, the low saturated fat diet was high in polyunsaturated rather than monounsaturated fatty acids. Under these conditions, no difference in glucose metabolism was observed.

Sarkkinen et al. [95] compared the effects of two diets in subjects with impaired glucose tolerance, one diet contained 46% of energy carbohydrates and 34% of energy fat and the other diet contained 42% of energy carbohydrates and 40% of energy fat. The amount of saturated fat was equal in both diets (11% of energy). Fasting blood glucose concentration and glucose effectiveness (an index derived from the results of intravenous glucose tolerance test) was significantly lower in the group that consumed the diet higher in fat.

Christiansen et al. [96] examined the effects of diets enriched either in saturated fat, *cis*-monounsaturated fat or *trans*-monounsaturated fat (20% of energy) on glucose metabolism in obese type 2 diabetic patients. Postprandial serum insulin and C-peptide responses were greater after consumption of the saturated and *trans*-monounsaturated fat enriched diets compared to the *cis*-monounsaturated fat enriched diet. Somewhat surprising, difference among the diets were found in fasting levels of serum lipids and lipoproteins.

In a recently published study, Lovejoy et al. [97] compared the effect of a high-fat diet containing 50% of energy as fat and 35% of energy as carbohydrate, and the low-fat diet containing 20% of energy as fat and 55% of energy as carbohydrate to a habitual diet on insulin sensitivity both in African–American and Caucasian women. Independent of race, the high-fat diet induced a significant reduction compared to a habitual diet in insulin sensitivity index (S_I) calculated by the minimal model method [98] from the results of frequently sampled intravenous glucose tolerance test. In contrast, the low-fat diet induced an increase in S_I, albeit not significant, in African–American women and a significant ($P < 0.04$) 20% increase in Caucasian women compared to a habitual diet.

Focusing on the effects of individual saturated fatty acids, Eckel and coworkers [99] compared the short term (5 days) effects of diets enriched in medium chain triglycerides (MCT) (40% fat of energy of which 77.5% MCT) or long chain triglycerides on type 2 diabetic patients and control subjects in a cross-over study. The MCT enriched diet was reported to have a favorable effect on insulin mediated glucose metabolism. Storm et al. [100] compared the effects of a high-fat diet (45% total fat (16% palmitic and 13% stearic acid diet), 40% carbohydrate) and a high-carbohydrate diet (51% carbohydrate, 29% fat) in type 2 diabetic patients. No difference in fasting blood glucose was observed.

Schwab et al. [101] have compared the effects of a 12% palmitic acid and 7% stearic acid (total fat 37%) on glucose homeostasis in healthy females. No difference in glucose metabolism measured by an intravenous glucose tolerance test between the diet periods was observed. The same workers [102] compared the effect of an 11% palmitic and 5% lauric acid diet in a similar setting again in healthy females. As for the earlier work [101], no difference in the results of the intravenous glucose tolerance test was observed between the diet periods.

Pan et al. [103] examined the effect of diet and/or exercise intervention in the prevention of the onset of type 2 diabetes in 577 subjects with impaired glucose tolerance. Over a 6-year period, normal weight participants in the diet or diet + exercise intervention group were prescribed a diet with 55–65% of energy as carbohydrate and 25–30% of energy as fat. There was no special attention to the quality of fat. Obese subjects were encouraged to loose weight. The diet intervention was associated with 31% reduction in risk of developing type 2 diabetes, whereas the diet + exercise intervention was associated with a reduction of 42%.

In summary of the in vivo metabolic studies, high fat diets with favorable fatty acid composition (high P/S ratios) appear to result in more favorable glycemic control compared to high carbohydrate diets in type 2 diabetic patients [68,70]. In studies where only the dietary fat level, not the fatty acid profile, was modified, the results have been less consistent. In some studies, a high carbohydrate diet negatively affected glycemic control [66,67] whereas in other studies no effect was observed [69,81]. In subjects with normal glucose metabolism, no difference between the high fat

diet and the high carbohydrate diet has been demonstrated [65,88]. However, high carbohydrate diets relative to high fat diets have been reported to cause unfavorable changes in lipid profile (increased triglycerides, decreased HDL cholesterol levels) [64–71,92].

Regarding the quality of fat there are fewer studies published, and for the most part were short term. This has hampered elucidation of mechanisms with regard to insulin sensitivity and fatty acid type. In summary, it can be concluded that substitution of unsaturated fat for saturated fat may have positive effect on glucose metabolism. In the very limited data regarding individual saturated fatty acids no difference among saturated fatty acids has been demonstrated.

3.2. Metabolic studies/in vitro

Ginsberg et al. [104] assessed the effects of media enriched in either a monounsaturated fatty acid, oleic acid (18:1), or a polyunsaturated fatty acid, linoleic acid (18:2), on binding and number of insulin receptors in erythroleukemia cells cultured for five generations. Cells cultured in both medias exhibited an increased number of low affinity insulin receptors and decreased receptor affinity relative to the control cells. The authors concluded that an increase in the saturated fatty acid content of the membrane led to a decrease in membrane fluidity, and number of low affinity insulin receptors and an increase in affinity of the low affinity receptors.

Borkman et al. [105] assessed the relationship of insulin sensitivity and fatty acid composition in skeletal muscle phospholipids. They reported that in patients with coronary artery disease, fasting serum insulin concentration was negatively correlated with the percent of long-chain polyunsaturated fatty acids (arachidonic acid) in the phospholipids fraction, independent of age, sex, adiposity, and type of therapy. Similar results were found in normal subjects between fasting insulin concentrations and the fatty acid phospholipid profile of muscle. An additional measure, insulin sensitivity, was positively correlated with phospholipid polyunsaturated fatty acids. It was concluded that the data are consistent with the hypothesis that the fatty acid composition of skeletal muscle phospholipid influences the sensitivity of the tissue to insulin. No significant relationship of either measure with the saturated fatty acid content of skeletal muscle was reported.

Vessby et al. [106] further assessed the effect of skeletal muscle phospholipid fatty acid profile on insulin sensitivity. Seventy-year-old men served as study subjects. Peripheral insulin sensitivity was significantly and negatively correlated with the proportion of palmitic, palmitoleic, and di-homo-γ-linolenic acids and consistent with that reported by Borkman et al. [105], and positively correlated with linoleic acid.

Baur et al. [107] examined the relationship of the fatty acid composition of skeletal muscle membrane phospholipid with the type of feeding of children younger than 2 years. The breast-fed infants had higher percentage of long-chain polyunsaturated fatty acids in muscle phospholipids and lower plasma glucose concentrations compared to the formula-fed group. A significant inverse correlation between fasting plasma glucose concentration and the percentage of long-chain polyunsaturated fatty acids in skeletal muscle membrane phospholipids was also reported.

In summary, in vitro data suggest that the fatty acid composition of the membranes of peripheral tissues affects insulin sensitivity. The possible mechanisms include the fluidity of cell membrane, the number and affinity of insulin receptors, and, as discussed in the Section 2 (animal data), changes in the activities of enzymes associated with glucose metabolism.

3.3. Epidemiological studies (population based)

Early work [57,108–112] suggested a population-wide relationship between diet, obesity, diabetes mellitus, and other degenerative disease associated with increased affluence. West et al. [113] reported that total fat, animal fat (a marker of saturated fat), and protein were positively related to the risk of developing diabetes and that there was an inverse association with carbohydrate intake.

Kawate et al. [114] compared Japanese people living in Hawaii or Japan and found that those living in Hawaii consumed twice the animal fat and simple carbohydrate, and had a significantly higher prevalence of diabetes than those living in Japan. Confounding factors include body mass index and level of physical activity. In a similar type of study focusing on Seventh-day Adventistis, Snowdon [115] reported a positive association between the incidence of type 2 diabetes and animal product consumption.

Feskens and Kromhout [116] demonstrated that in normal subjects saturated fatty acid intake was positively correlated with fasting glucose concentrations, whereas fiber intake was inversely correlated with glucose area under the curve after an oral glucose tolerance test. No data on insulin levels were presented. In a more recent publication of the same cohort this group reported that insulin concentrations during the oral glucose tolerance test were inversely associated with the dietary intake of polyunsaturated fat and positively associated with the intake of saturated fat [117]. This group has also investigated the role of diet as a predictor of the onset of type 2 diabetes [118]. The intake of total, saturated and monounsaturated fat 20 years before the diagnosis was higher in men with newly diagnosed diabetes compared to men with normal or impaired glucose tolerance. The past intake of total fat

was also reported to be associated positively with the 2 h post-load glucose level. The intake of total fat and monounsaturated fat were correlated in the Western diet and may explain the positive association of monounsaturated fat and the onset of type 2 diabetes reported in this study.

Trevisan et al. [119] reported a cross-sectional association between the intake of various dietary fats and risk factors for coronary heart disease in a cohort of Italian men and women. Consumption of butter was positively associated with blood glucose concentrations both in men and women, whereas consumption of olive oil and other vegetable oils was inversely associated with blood glucose concentrations in both genders.

Salomaa et al. [120] assessed the relationship between dietary fatty acid intake and glucose tolerance in normal and diabetic subjects. There was a positive correlation between the proportion of palmitic and palmitoleic acids in plasma cholesteryl esters and glucose tolerance. Diabetic individuals had a higher intake of saturated fatty acids than control subjects. The authors postulated that alterations in the fatty acid composition of membrane lipids may be associated with insulin resistance and blood glucose regulation.

Tsunehara et al. [121] explored the relationship between diet and the high rates (four-fold increased) of glucose intolerance in second-generation Japanese–American men. They reported that those men with glucose intolerance had higher intakes of animal protein and fat, yet no difference in caloric intake. A similar relationship was reported by Dahlquist et al. [122], between children with and without insulin-dependent diabetes. Although a relationship between protein and diabetes was reported the authors stated that "Most nutrients classified as rich in protein are also rich in fat".

Marshall et al. [123] investigated the relationship of diet and risk for type 2 diabetes using data from two counties in southern Colorado. Increased risk of type 2 diabetes and impaired glucose tolerance was related to increased intake of fat. No data on the fatty acid profile of the diet was presented.

Maron et al. [124] addressed the potential association between diet and plasma insulin concentrations in non-diabetic men with cardiovascular disease. They reported that after adjusting for age, the intake of saturated fat and cholesterol were positively correlated with fasting insulin concentrations, body mass index, and waist-to-hip ratio. Multivariate analysis indicated that intake of saturated fat was significantly related to fasting insulin concentrations, independent of body mass index. These data support similar work in animals.

Lovejoy and DiGirolamo [125] investigated the potential association between habitual dietary intake and insulin sensitivity in lean and obese subjects. Percent of energy intake as fat was positively correlated with body mass index and diminished insulin sensitivity (the opposite was true for fiber intake). There was no association of P/S ratio with body mass index or insulin sensitivity.

Colditz et al. [126] related the risk of developing clinical type 2 diabetes and diet in a large cohort of women participating in the Nurses' Health Study. Using a food frequency questionnaire, after controlling for body mass index, they reported that vegetable fat or linoleic acid intake was inversely related to the risk of developing type 2 diabetes. Animal fat intake was weakly, but not statistically, related to risk of developing type 2 diabetes. However, there was a strong inverse relationship between P/S ratio of the diet and risk of developing type 2 diabetes.

Parker et al. [127] assessed the relationship between diet, and fasting and postprandial insulin concentrations among individuals aged 43–85 years as part of the Normative Aging Study. Log-transformed fasting insulin concentrations were positively associated with saturated fatty acid intake, in addition to body mass index, abdomen–hip ratio, and total fat intake. Multivariate models indicated that saturated fatty acid intake, body mass index and abdomen–hip ratio were independent predictors of both fasting and postprandial insulin concentrations after adjusting for age, cigarette smoking, and physical activity. It was estimated that if saturated fatty acid intake (% of calories) were decreased from 14 to 8%, there would be an 18% decrease in fasting insulin and a 25% decrease in postprandial insulin concentrations. Drawing from the data generated from animal models, the authors proposed the insulin resistance observed was related to increases in the saturated fatty acid content of plasma membranes. With respect to methodology, it was felt that any error in assessing food intake using food frequency questionnaires would be biased against fat, thereby underestimating the effect.

Shimakawa et al. [128] related dietary fat intake to hemoglobin A_1 levels, used as a measure of glycemic control, in individuals with type 2 diabetes. In males, but not females, % of energy consumed as fat was significantly correlated with hemoglobin A_1 levels. No data on the fatty acid profile of the diet was available.

Mayer et al. [129] assessed the relationship between dietary fat intake, and fasting and postprandial insulin levels. They concluded that an increase in the intake of dietary fat was associated with an increase in fasting insulin concentrations. Saturated fat intake was significantly associated with the 2 h postglucose load insulin concentration before, but not after, adjustment for body weight. Similarly, within identical twin pairs, total dietary fat was positively related to fasting insulin before, but not after, adjustment for body weight. It was unclear whether there was an independent relationship of dietary fat intake and hyperinsulinemia, or whether it was related to obesity.

South Asian men settling in Europe exhibit high rates of type 2 diabetes and coronary disease. Sevak et al. [130] investigated this trend by focusing on diet, and fasting and postprandial insulin concentrations, in South Asian men and compared these parameters to European men. As a % of total energy, South Asians had lower total and saturated fat, and higher monounsaturated fat, polyunsaturated fat, carbohydrate, and alcohol intakes than European men. Total or saturated fatty acid intake were not related to fasting or 2 h post-glucose load insulin concentrations. Insulin concentrations were positively related to carbohydrate intake and inversely related to alcohol intake. The standard dietary risk factors for the development of hyperinsulinemia could not account for the differences observed between the two populations studied.

Marshall et al. [131] examined the effect of dietary fat intake on the development of type 2 diabetes in subjects with impaired glucose tolerance. The subjects were followed for 11–40 months (on average 22.6 months). The mean percentage of energy from fat was significantly higher in those subjects who developed type 2 diabetes (43.4%) compared to those who continued to have impaired glucose tolerance (40.6%) or those whose glucose tolerance reverted to normal (38.9%). After adjustment for energy intake, age, sex, ethnicity, and obesity, an increase in fat intake of 40 g/day was associated with a 3.4-fold increase in risk of type 2 diabetes. The results of the Insulin Resistance Atherosclerosis Study (IRAS) also suggested that a high intake of dietary fat may worsen glucose metabolism [132]. The subjects in this study were obese subjects who are already at increased risk for type 2 diabetes.

Vessby et al. [133] investigated whether the risk to develop type 2 diabetes among 50-year-old men was related to the fatty acid composition of serum cholesteryl esters. The follow-up period was 10 years. Those who developed type 2 diabetes had higher proportions of saturated fatty acids, palmitoleic acid, and γ-linolenic and dihomo- γ-linolenic acids, and lower proportion of linoleic acid in serum cholesteryl esters compared to those who did not develop type 2 diabetes. The authors suggested that the reason for the higher proportion of γ-linolenic and dihomo-γ-linolenic acids in those who developed type 2 diabetes may have been a result of changed activities of enzymes in fatty acid metabolism, e.g. Δ-5 and Δ-6 desaturases.

Bell et al. [134] assessed dietary intake and glycemic control (glycosylated hemoglobin A_{1c}, $GHbA_{1c}$) in a racially mixed population of adults with type 2 diabetes. Total energy intake predicted $GHbA_{1c}$ levels in all subjects. Dietary fat had a positive association with glycemic control among black subjects. No association was seen for individual fatty acids or fatty acid subclasses.

The epidemiological data suggest that diets associated with affluence or Western countries are associated with a higher incidence of type 2 diabetes. That is, there appears to be a pattern suggesting a clustering of high intakes of dietary fat (especially animal fat), obesity and glucose intolerance. Clearly, there are many issues that impact on these observations, both dietary (e.g. type of carbohydrate — simple or complex, glycemic index) and environmental (e.g. level of physical activity). It is virtually impossible to separate them out in a meaningful way. However, regarding these issues, type of fat itself becomes somewhat moot. Saturated fat (with the notable exception of stearic acid) results in increased plasma lipid and lipoprotein levels. Since diabetes is an independent risk factor for the development of coronary heart disease, one would recommend to individuals with diabetes weight loss and adopt a Step 1 diet ($\leq 30\%$ fat, $< 10\%$ saturated fat, < 300 mg cholesterol). If inadequate response occurs, a Step 2 diet ($\leq 30\%$ fat, $< 7\%$ saturated fat, < 200 mg cholesterol) [135].

In conclusion, the evidence from animal studies suggests that a diet high in fat affects glucose metabolism negatively. In humans (mostly type 2 diabetic patients) the results have been inconsistent and are likely confounded by differences in body weight. In studies in which the fatty acid composition of a high-fat diet has been modified to contain a higher proportion of unsaturated fat the high-fat diet has improved glucose metabolism compared to a high-carbohydrate diet. Epidemiological data suggest that subjects with higher intakes of fat are more prone to develop disturbances in glucose metabolism, type 2 diabetes or impaired glucose tolerance, than subjects with lower intake of fat. These data are confounded by differences in body weight.

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Effect of a High-Monounsaturated Fat Diet Enriched With Avocado in NIDDM Patients

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OBJECTIVE— To assess the effects of two controlled diets, one rich in oleic acid obtained from avocado and olive oil and the other rich in complex carbohydrates, on fasting and postprandial serum lipids and glycemic control in 12 women with NIDDM.

RESEARCH DESIGN AND METHODS— A randomized crossover study was designed. During a 4-week baseline period, all patients received the isocaloric diets recommended by the American Diabetes Association. After this period the patients were randomly assigned to receive the two study diets alternately during two 4-week periods. One diet was high in monounsaturated fatty acids (HMUFA) and the other was high in complex carbohydrates (high-CHO). There also was a 4-week washout period in between the two 4-week periods during which the patients followed the American Diabetes Association's isocaloric diet. Blood samples were obtained before and after each dietary period.

RESULTS— Both diets had a minor hypocholesterolemic effect with no major changes in high-density lipoprotein cholesterol. The HMUFA diet was associated with a greater decrement in plasma triglycerides (20 vs. 7% in the high-CHO diet). Glycemic control was similar with both diets.

CONCLUSIONS— Partial replacement of complex digestible carbohydrates with monounsaturated fatty acids (avocado as one of its main sources) in the diet of patients with non-insulin-dependent diabetes mellitus improves the lipid profile favorably, mantains an adequate glycemic control, and offers a good management alternative.

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NIDDM, non-insulin-dependent diabetes mellitus; HMUFA diet, high–monounsaturated fatty acids diet; high-CHO diet, high-carbohydrate diet; LDL, low-density lipoprotein; HDL, high-density lipoprotein; CV, coefficient of variation.

Dietary treatment represents the cornerstone in the management of diabetes (1). High-carbohydrate, low-fat diets are currently recommended because they consistently lower low-density lipoprotein (LDL) cholesterol (1–5). However, diabetic dyslipidemia frequently involves hypertriglyceridemia and/or hypo-α-lipoproteinemia (6). For this reason, many authors have suggested modifications to these dietary guidelines, particularly that monounsaturated fats be used as a better replacement for saturated fatty acids than carbohydrates. An excessive amount of carbohydrates in the diet can, in certain groups of patients, contribute to a more marked hypertriglyceridemia and/or postprandial hyperglycemia (7–12).

Foods containing monounsaturated fats are scarce, and most studies have been limited to the use of olive oil. Avocado has a high content of monounsaturated fatty acids (oleic acid), and large quantities of this fruit are consumed in Mexico and some other countries. The purpose of this study was to compare the effects of a high-carbohydrate (CHO) diet with a high–monounsaturated fatty acids (HMUFA) diet (a significant portion of which was derived from avocado) on glycemic control and the lipid and lipoprotein profile of patients with non-insulin-dependent diabetes mellitus (NIDDM).

RESEARCH DESIGN AND METHODS

— Sixteen female patients with NIDDM were studied at the National Institute of Cardiology Ignacio Chávez Endocrinology Department. All had a previous diagnosis of NIDDM based on the National Diabetes Data Group criteria. The patients had good metabolic control (mean fasting blood glucose levels <7.77 mM) and no major complications related to diabetes. Most of them were on oral hypoglycemic agents associated with diet, and none had previously received insulin. Some had hypertension and/or atherosclerotic heart disease and were on diuretics, ACE (angiotensin-converting

Table 1—*Mean composition of the study diets*

	ADA diet	HMUFA diet	High-CHO diet
Total fat (% of total energy)	30	40	20
Saturated	10	11	6.6
Monounsaturated	10	24	6.6
Polyunsaturated	10	5	6.6
P:S ratio	1	0.45	1
Carbohydrates (% of total energy)	55	40	60
Proteins (% of total energy)	15	20	20
Cholesterol (mg/day)	<300	<300	<300

Both diets were isocaloric, the fiber content was ~30 g in the HMUFA diet and 42 g in the high-CHO diet. ADA, American Diabetes Association; P:S, polyunsaturated:saturated.

enzyme) inhibitors, or calcium channel blockers, which were continued with no changes during the study. None had had a recent occurrence of myocardial infarction, unstable angina, or congestive heart failure. Also, none of the patients had thyroid, renal, or hepatic disease or had received a hypolipidemic agent in the 3 months before the study. Of the original 16 patients, only 12 are included in this report. Their mean ± SD age was 56 ± 8 years, and the body weight and body mass indexes averaged 66 ± 10 kg and 28 ± 4 kg/m^2, respectively.

A randomized crossover study was designed. During a 4-week baseline period, the patients received the isocaloric diets recommended by the American Diabetes Association. After this period, the patients were randomly assigned to alternately receive both study diets—the HMUFA and high-CHO diets—during two 4-week periods with a 4-week period in between during which the patients were kept on the American Diabetes Association's isocaloric diet. Four patients were excluded because they did not comply with the dietary guidelines.

Diets

The nutrient composition of the diets is shown in Table 1. Both diets consist of solid foods, and patients were allowed some choice of food items. One avocado (Hass variety) and four teaspoons of olive oil were the main sources of fat in the HMUFA diet.

The patients were instructed to follow the diet and received menus every day (Table 2). On the 14th day and at the end of each study period, the patients were seen by the nutritionist and had a 24-h diet recall. Four patients had <80% adherence to the diet (one with >5% change in her body weight) and were excluded from the study.

At the beginning and end of each period, a mixed meal consumed in 20 min was given as a provocative test (550 kcal with the distribution pattern of each of the study diets), and the postprandial increments in insulin, glucose, and triglycerides were measured.

Biochemical analysis

On the first and last day of each period, 12- to 14-h fasting blood samples were obtained and analyzed for total cholesterol, triglycerides, LDL and high-density lipoprotein (HDL) cholesterol, glucose, insulin, and fructosamine. After the provocative mixed-meal test, glucose, insulin, and triglycerides also were measured at the first, second, and sixth hour.

Total cholesterol and triglyceride levels were determined by enzymatic methods (13,14). HDLs, including HDL_3 levels, were determined after double precipitation using $MgCl_2$ and dextran-sulfate (15). LDL-cholesterol concentration was estimated using the Friedewald equation. Laboratory methods were standardized through the National Heart, Lung, and Blood Institute–Center for Disease Control lipoprotein standardization program. Intra-assay coefficients of variation (CVs) for total cholesterol, triglycerides, and HDL cholesterol were 1.1, 0.62, and 1.14%, respectively; the interassay CVs were 3.06, 2.6, and 3.9%, respectively. Blood glucose was determined by a glucose oxidase method with commercially available kits (Test-Combination glucose GOD-PAP, Boehringer-Mannheim, Mannheim, Germany).

Fructosamine was assayed with a method that measures the reduction of nitro blue tetrazolium by serum (test-combination fructosamine, Boehringer-Mannheim) with an ABBOTT VP II analyzer. Plasma insulin was measured by enzyme-linked immunosorbent assay with commercially available kits (Enzymun-Test Insulin, Boehringer-Mannheim).

Statistical analysis

To compare the baseline periods and the two study periods, Wilcoxon's signed-rank test was used. Data are expressed as means ± SD. Differences in the postprandial metabolic variables were analyzed with their percentage changes (% Δ). Analysis was performed, when required, on the logarithms of the triglycerides to improve their skewed distribution. These variables were then converted into their natural units for presentation in the tables.

RESULTS— The effect of the two different diets on the lipid profiles and diabetic metabolic control is shown in Table 3 and Fig. 1. Both diets had a similar and minor hypocholesterolemic effect, with no major changes in HDL cholesterol. The HMUFA diet was associated with a greater decrement in plasma triglycerides (20 vs. 7% in the high-CHO diet, NS). Figure 2 shows the individual triglycerides values before and after the two dietary periods. As can be seen, the major hypotriglyceridemic effect was obtained in patients with basal higher triglyceride values when they received the HMUFA diet. The high-CHO diet also was associ-

Table 2—*Example of a menu*

HMUFA diet*‡		High-CHO diet†‡	
Breakfast		Breakfast	
Coffee with nonfat milk	240 ml	Coffee with nonfat milk	240 ml
Enfrijolada		Entomatadas	
1 corn tortilla		3 corn tortillas	
1/2 cup beans		tomato sauce	
1/3 piece avocado		onion, lettuce	
Guavas	2 pieces	Papaya	3/4 cup
Lunch		Lunch	
Rice with vegetables	1/2 cup	Potato soup	1 1/2 cups
Roast meat	120 g	Spanish rice	1/2 cup
Beans in a bowl	1 cup	Chili with chicken and nopales	100 g
Corn tortilla	1 piece	Corn tortillas	3 pieces
Guacamole			
1/3 piece avocado			
onion, tomato			
Tangerine	1 piece	Apple	1 piece
Lemonade		Lemonade	
Dinner		Dinner	
Taco (with cooked vegetables)		Beans in a bowl	1 cup
corn tortilla	1 piece	Bread	2 slices
1/3 piece avocado			
Apple	1 piece	Orange	1 piece
Tea		Tea	

Operational definitions: tortilla: baked, flat, round, thin cakes of unleavened cornmeal (masa) or flour—the bread of México; enfrijolada: corn tortilla folded or rolled, covered with a bean sauce and garnished with onion and lettuce; entomatada: corn tortilla folded or rolled, covered with sauce of chili with tomato, and garnished with onion and lettuce; guacamole: avocado dip made with chopped chili, tomato, onion, and other seasonings; taco: corn tortilla folded in half to hold seasoned beef, beans, or vegetables; nopales (cactus): the leaves or pods of the prickly pear cactus are used; they taste like crisp green beans and are sliced in strips and cooked with onions and spices. *Four teaspoons of olive oil used for cooking. †Three teaspoons of safflower oil used for cooking. ‡Drinks could be sweetened with artificial edulcorants.

ated with minor decrements in the level of triglycerides.

The provocative mixed-meal test at the end of each experimental diet showed that the differences between the HMUFA and the high-CHO diets mainly related to the fasting glucose, insulin, and/or triglyceride levels; all three parameters diminished after each experimental diet. The percentage changes (% Δ) were otherwise similar. Although the differences were not statistically significant, the trend was toward higher postprandial glucose values after a high-CHO meal test, likely related to the fact that more carbohydrates were eaten during this meal test.

CONCLUSIONS— The study was designed to assess whether replacing carbohydrates with monounsaturated fats, most of them derived from avocado and olive oil, would induce beneficial changes in the lipoprotein patterns in patients with NIDDM.

The Mexican diet in the lower income classes is high in carbohydrates (± 60%); the amount of fat is relatively low (± 20%), but most of it is saturated and accompanied by a high amount of cholesterol. This meal pattern plus the genetic admixture and high prevalence of obesity probably explain the high prevalence of NIDDM and lipid disorders in the Mexican populations, particularly hyper-

Table 3—*Effects of the two study diets on plasma lipids, lipoproteins, and glycemic control*

	HMUFA diet			High-CHO diet		
	Before	After	*P* value	Before	After	*P* value
Glycemia (mM)	6.49 ± 1.11	5.43 ± 1.11	<0.001	7.16 ± 2.6	6.16 ± 1.38	<0.05
Fructosamine (mM)	271 ± 79	270 ± 47	NS	284 ± 67	272 ± 50	NS
Weight (kg)	65 ± 10	64 ± 10	NS	65 ± 10	65 ± 10	NS
Total cholesterol (mM)	5.22 ± 0.93	4.83 ± 0.88	<0.05	5.30 ± 0.80	5.07 ± 1.11	<0.05
LDL cholesterol (mM)	3.5 ± 0.75	3.38 ± 0.72	NS	3.69 ± 0.70	3.48 ± 0.88	NS
HDL cholesterol (mM)	0.98 ± 0.23	0.98 ± 0.26	NS	0.96 ± 0.20	0.98 ± 0.26	NS
Triglycerides (mM)	1.75 ± 0.96	1.25 ± 0.41	<0.05	1.78 ± 0.75	1.61 ± 0.68	NS

P values obtained by Wilcoxon's signed-rank test. When the different variables in the HMUFA vs. the high-CHO diets were compared, nonsignificant differences were observed between baseline values. After both dietary periods, significant differences ($P < 0.05$) were shown only with plasma triglycerides.

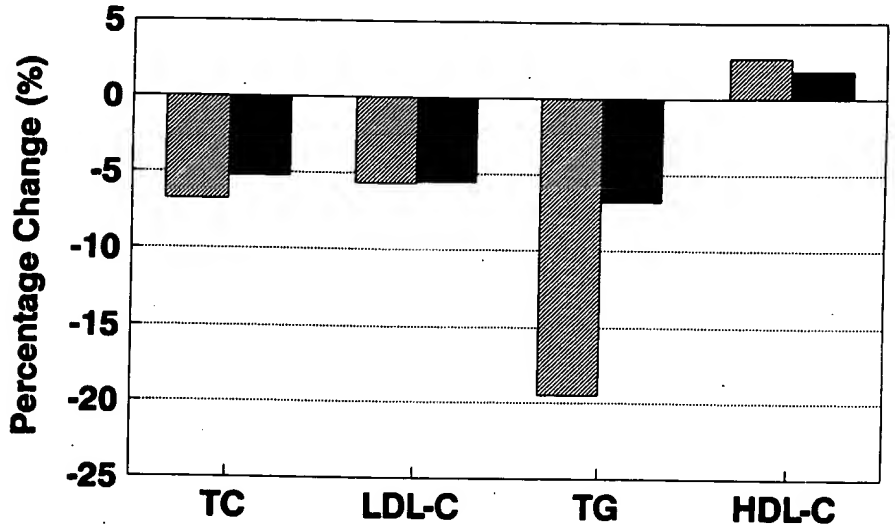

Figure 1—*The percentage of change in blood lipids after the HMUFA (▨) and high-CHO (■) diets in 12 NIDDM patients. (TC), Total cholesterol; (LDL-C), LDL cholesterol; (TG), triglycerides; (HDL-C), HDL cholesterol.*

triglyceridemia and hypo-α-lipoproteinemia.

Avocado is among the fruits commonly used in the Mexican diet. Because it is rich in fat, avocado has not been recommended for individuals with dyslipidemias. For every 100 g of edible portion of avocado (Hass, a subtype of avocado), 144 kcal are distributed with 7.6 g of carbohydrates, 1.6 g of protein, and 11.9 g of total fat. Of this, 15.2% are saturated, 72.6% are monounsaturated (mostly oleic acid), and 12.2% are polyunsaturated with a polyunsaturated:saturated fat ratio of 0.8 (16,17). During the last few years, however, some brief reports have suggested that avocado consumption probably has no detrimental effect on the lipid profile.

In this study, both diets were well tolerated with no adverse effects, and excellent compliance was attained, probably because patients were highly motivated and had day-by-day menu selection. This is confirmed by the expected dietary reduction in total cholesterol of 5–6%, in agreement with the Keys equation, as formulated by Anderson et al. (18a).

Overall, the HMUFA diet, supplemented with avocado, showed results similar to those obtained in other studies with greater amounts of olive oil. The reduction in serum triglycerides was significant, however, the decrements in total cholesterol were only minor, and no change was observed in HDL cholesterol (7–12,18). The observation that individuals with the higher basal triglyceride values obtained significant decrements on their triglyceride levels with the HMUFA diet was particularly important. To reinforce the fact that triglyceride levels, and not order effect, were relevant to the results obtained, we present the mean triglyceride values before and after each dietary period for both groups of patients (A1, A2 and B1, B2; Fig. 3). Both diets had a hypotriglyceridemic effect, but it was only shown in the patients who had higher basal triglyceride values (group A1, A2). The group (B1, B2) with lower baseline triglyceride values had basically no changes with both diets. The study was performed in a randomized fashion, so the fact that one group of patients had higher basal triglycerides cannot be explained. Both groups of patients were similar in age, type of treatment, metabolic control, and duration of diabetes. These data do not suggest an order effect. The absence of a greater response with the HMUFA diet in the second period is likely related to the lower baseline triglyceride values. In summary, people did not do better if one diet was started first or second. The hypotriglyceridemic effect was more related to the baseline triglyceride values and reached statistical significance only for the HMUFA diet.

Potentially undesirable effects of high-CHO diets, such as reductions in

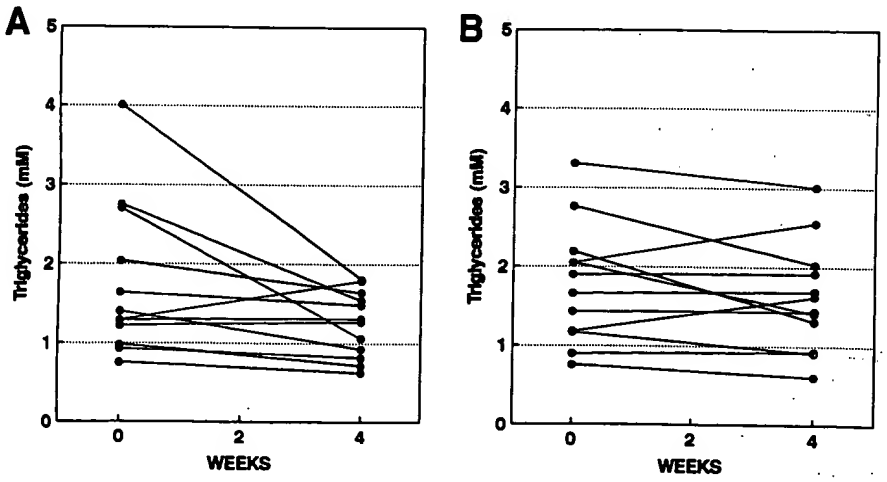

Figure 2—*Individual changes in plasma triglyceride values after the HMUFA (A) and high-CHO (B) diets.*

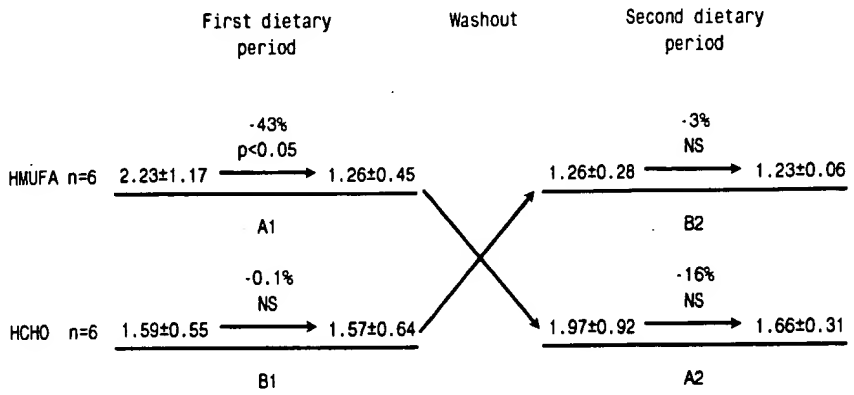

Figure 3—*Mean triglyceride values before and after each dietary period for both groups of patients (A1, A2 and B1, B2).*

HDL cholesterol, hyperglycemia, and/or hypertriglyceridemia (7,8,19,20), were not seen in this study. A plausible explanation is that a deleterious hyperglycemic and hypertriglyceridemic effect of these types of diets is generally found in individuals with poor metabolic control. This was not the case in this study. We could not find significant differences in the postprandial increments of triglycerides, glucose, and/or insulin levels with either diet. No differences were found in the blood glucose control between both types of diets.

In conclusion, partial replacement of complex digestible carbohydrates with monounsaturated fatty acids (avocado as one of its main sources) in the diet of patients with NIDDM favorably improves both lipid profiles, mantains an adequate glycemic control, and should be a good management alternative. Further studies are required regarding the long-term effects of the avocado.

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Dietary Fat and Incidence of Type 2 Diabetes in Older Iowa Women

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OBJECTIVE — To examine the associations between reported intakes of dietary fat and incident type 2 diabetes.

RESEARCH DESIGN AND METHODS — We studied the relation between dietary fatty acids and diabetes in a prospective cohort study of 35,988 older women who initially did not have diabetes. Diet was assessed with a food frequency questionnaire at baseline, and 1,890 incident cases of diabetes occurred during 11 years of follow-up.

RESULTS — After adjusting for age, smoking, alcohol consumption, BMI, waist-to-hip ratio, physical activity, demographic factors, and dietary magnesium and cereal fiber, diabetes incidence was negatively associated with dietary polyunsaturated fatty acids, vegetable fat, and *trans* fatty acids and positively associated with ω-3 fatty acids, cholesterol, and the Keys score. After simultaneous adjustment for other dietary fat, only vegetable fat remained clearly related to diabetes risk. Relative risks across quintiles of vegetable fat intake were 1.00, 0.90, 0.87, 0.84, and 0.82 ($P = 0.02$). Diabetes risk was also inversely related to substituting polyunsaturated fatty acids for saturated fatty acids and positively correlated to the Keys dietary score.

CONCLUSIONS — These data support an inverse relation between incident type 2 diabetes and vegetable fat and substituting polyunsaturated fatty acids for saturated fatty acids and cholesterol.

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Although a low-fat diet is recommended for diabetic and nondiabetic patients (1), findings from epidemiological studies on the association of total dietary fat with type 2 diabetes or insulin sensitivity have been inconsistent (2–8). Metabolic and epidemiological studies suggest that dietary fat subtypes may be relevant to diabetes pathophysiology. Specific dietary fatty acids may influence the development of diabetes by modifying the phospholipid composition of cell membranes, which in turn may alter the function of the insulin receptor (9,10).

While controlling for dietary and nondietary factors, we examined the relation between baseline intake of total dietary fat and dietary fat subtypes and the development of type 2 diabetes over 11 years of follow-up in the Iowa Women's Health Study.

RESEARCH DESIGN AND METHODS

The Iowa Women's Health Study is a prospective cohort study of older Iowa women. In January 1986, a random sample of 99,826 women aged 55–69 years who had a valid Iowa driver's license were mailed a 16-page questionnaire and asked to participate. The study sample consisted of the 41,836 women who returned the baseline questionnaire. Respondents had a lower mean BMI (0.4 kg/m^2 less), were 3 months older, and were more likely to live in counties that were rural and less affluent than nonrespondents (11).

Women were excluded from analysis if they reported implausibly high (>5,000 kcal) or low (<600 kcal) energy intakes, left ≥30 items blank on the food-frequency questionnaire, or had diabetes at baseline. Women were considered to have diabetes at baseline if they responded "yes" or "don't know" to one of the following questions: 1) have you ever been told by a doctor that you have sugar diabetes? and 2) have you ever taken insulin or pills for sugar diabetes (or to lower blood glucose)? After exclusions, 35,988 women remained eligible for the study.

Data collection

The baseline questionnaire included questions on known or suspected risk factors for diabetes, such as age, BMI, waist-to-hip ratio (WHR), physical activity, alcohol consumption, and smoking history. BMI was calculated from weight and height measurements provided by the participants. WHR was calculated as the average of two measurements taken by the participant's spouse or friend using a paper tape measure that was included with the questionnaire (12). The women reported their frequency of moderate (e.g., golf and long walks) and vigorous (e.g., swimming and aerobics) physical activity. Pack-years of smoking were calculated from information on the intensity and duration of cigarette smoking. Alcohol consumption was assessed with a food frequency questionnaire that queried the participants' typical intakes of wine, beer, and spirits. In addition, the participants provided information on their marital status, educational attainment, residence, and use of hormone replacement therapy.

The principal dietary exposure of interest was intake of fat, including cholesterol and the Keys score. In addition to total dietary fat, we analyzed saturated, polyunsaturated, monounsaturated, *trans* fatty acids, long-chain ω-3 fatty acids, and

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Abbreviations: IGT, impaired glucose tolerance; RR, rate ratio; WHR, waist-to-hip ratio.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Distribution of baseline risk factors for diabetes according to energy-adjusted* dietary fat intake among 35,988 Iowa women, 1986–1997*

	Total fat		Saturated fatty acids		Poly-unsaturated fatty acids		Mono-unsaturated fatty acids		Long-chain ω-3 fatty acids		*Trans* fatty acids		Cholesterol		Animal fat		Vegetable fat	
Quintiles of intake:	1	5	1	5	1	5	1	5	1	5	1	5	1	5	1	5	1	5
Mean																		
Age (years)	61.9	61.1	61.9	61.1	61.6	61.5	61.8	61.1	61.6	61.4	61.6	61.6	62.0	61.1	61.9	61.0	61.5	61.5
BMI (kg/m^2)	26.2	27.2	26.1	27.0	26.6	26.9	26.2	27.2	26.4	27.4	26.4	26.8	25.9	27.7	26.0	27.3	26.9	26.6
WHR	0.830	0.840	0.829	0.839	0.836	0.834	0.830	0.840	0.837	0.833	0.830	0.838	0.831	0.841	0.829	0.840	0.837	0.834
Percent																		
Never drinker	49.2	57.8	48.2	57.3	53.4	51.6	48.9	58.7	59.2	49.6	47.3	59.6	52.7	55.2	50.2	56.9	51.9	54.6
High-school graduate	83.3	79.9	83.5	78.6	80.5	83.3	84.0	80.0	79.2	84.0	84.4	80.3	82.6	79.9	83.5	78.9	80.7	83.3
High physical activity	33.8	18.6	33.0	19.0	27.0	24.3	35.8	17.3	21.5	31.8	36.4	17.3	27.2	24.0	30.6	20.0	28.8	21.1
Current smoker	12.9	20.4	13.0	21.1	15.3	16.2	12.6	19.7	15.6	14.2	14.5	16.1	14.5	17.3	13.8	19.0	15.2	16.8
Never used hormone replacement therapy	60.2	63.1	58.6	64.3	63.6	59.1	59.5	63.8	62.2	59.7	58.2	63.4	58.8	63.8	58.2	64.6	63.5	59.2
Currently married	74.4	81.0	75.2	80.2	77.5	77.4	74.5	81.7	78.5	75.8	76.6	77.2	74.9	79.5	73.7	82.6	79.2	75.4
Urban residence (>10,000 population)	38.9	28.9	39.1	28.9	31.4	36.9	39.3	28.7	30.2	37.6	39.1	31.3	38.4	29.0	40.5	26.1	29.9	37.6
Correlation coefficient																		
Total fat	1.0		0.82		0.47		0.96		−0.16		0.50		0.35		0.67		0.40	
Saturated fatty acids			1.0		−0.03		0.70		−0.20		0.25		0.40		0.86		−0.06	
Polyunsaturated fatty acids					1.0		0.43		0.06		0.33		−0.07		−0.23		0.86	
Monounsaturated fatty acids							1.0		−0.19		0.62		0.29		0.60		0.43	
Long-chain ω-3 fatty acids									1.0		−0.23		0.20		−0.07		−0.11	
Trans fatty acids											1.0		−0.05		0.08		0.52	
Cholesterol													1.0		0.58		−0.30	
Animal fat															1.0		−0.42	
Vegetable fat																	1.0	

*Adjusted for total energy intake according to the methods of Willett and Stampfer (19).

animal and vegetable fat. The Keys score, which correlates changes in fatty acid intake with changes in serum cholesterol, was calculated as $1.26\,(2S - P) + (Z)^{1/2}$, where S = percent of energy from saturated fat, P = percent of energy from polyunsaturated fat, and Z = dietary cholesterol in mg/1,000 kcal (13). A higher Keys score corresponds to higher relative intake of saturated fat and cholesterol and a lower relative intake of polyunsaturated fat. We also examined several foods and food groups high in dietary fats, including total meat, red meat, eggs, nuts, and olive oil salad dressing.

A 127-item food-frequency questionnaire similar to that used in the 1984 Nurses' Health Study was used to assess typical food intake over the previous year (14). The validity of the food-frequency questionnaire was evaluated by comparing nutrient values determined from the questionnaire with values estimated from the average of five 24-h dietary recall surveys in 44 study participants (15). Energy-adjusted Pearson's correlation coefficients were 0.62, 0.59, 0.62, 0.43, and 0.21 for total fat, saturated fat, monounsaturated fat, polyunsaturated fat, and cholesterol, respectively. Correlation coefficients were not calculated for other fat variables.

Diabetes incidence was determined by an affirmative response to the following question, which was on all of the follow-up mailed surveys: "Since baseline or respective follow-up, were you diagnosed for the first time by a doctor as having sugar diabetes?" During 11 years of follow-up, 1,890 women reported incident diabetes in the four follow-up surveys administered in 1987 (n = 344), 1989 (n = 331), 1992 (n = 466), and 1997 (n = 749). Response rates for the four follow-up surveys were 91, 89, 86, and 79%, respectively.

A validation study of self-reported diabetes was conducted on 85 cohort participants in 1988 after the first follow-up survey. Subjects tended to over-report having diabetes; of 44 women who reported having diabetes at baseline, 28 (64%) were confirmed as having diabetes by their physician. All 41 women who reported not having diabetes at baseline were confirmed as not having diabetes (16).

Statistical analysis

For women who did not report a diagnosis of diabetes, person-time-at-risk was

calculated from baseline to the date of the last completed follow-up survey. For women who reported a diagnosis of diabetes, person-time was calculated as the sum of the known disease-free period and half of the period during which the diagnosis was made. Mortality status was determined annually through linkage with the State Health Registry of Iowa or, in the case of nonrespondents and emigrants from Iowa, via the National Death Index.

Nutrient intakes were adjusted for total energy through the residual method (17) and divided into quintiles. Trend analyses weighted each category of dietary intake by the median intake for that category. Cox proportional hazards regression models provided estimates of rate ratios (RRs). The SAS package was used (18); all *P* values were two-sided.

To examine the independent effects of specific fat subtypes, we simultaneously adjusted for all fat subtypes. Because we adjusted for dietary protein, the regression coefficients are an estimate of the effect of substituting a specific fat subtype for carbohydrates in the diet. We further examined substituting one fat subtype for another by including total fat and all fat subtypes except the subtype for which we substituted (19).

RESULTS — The distributions of several known risk factors for diabetes and correlation coefficients among dietary fat variables are presented in Table 1. Women in the highest category of saturated, monounsaturated, *trans* fatty acids, and animal fat had a higher BMI and WHR, but they consumed less alcohol and engaged in less physical activity. For cholesterol, these relations were similar but not as pronounced. The risk-factor differences among categories of polyunsaturated and ω-3 fatty acids and vegetable fat were not extreme. Overall, 99% of the population was non-Hispanic white and 95% were of Protestant or Catholic religious faith; these distributions did not vary according to fat intake. Positive correlations were evident between total and saturated fat, total and monounsaturated fat, animal and saturated fat, and vegetable and polyunsaturated fat.

After adjusting for nondietary factors in a multivariate regression analysis, total dietary fat, saturated fatty acids, and monounsaturated fatty acids were not related to incident diabetes (Table 2). Animal fat, cholesterol, the Keys score, and ω-3 fatty acids were positively correlated to diabetes. Comparing the highest with the lowest levels of intake, animal fat was associated with a 20% increase in incident diabetes (RR 1.19 and 95% CI 1.02–1.39). Comparing the highest with the lowest quintiles of intake, diabetes incidence increased with dietary cholesterol intake and the Keys score (1.24, 1.07–1.43) and (1.27, 1.08–1.49), respectively. There were inverse relations with polyunsaturated fatty acids and *trans* fatty acids and vegetable fat. Relative risks among quintiles of intake were 1.0, 0.93, 0.90, 0.84, and 0.87 ($P = 0.03$) and 1.0, 0.88, 0.84, 0.81, and 0.78 ($P = 0.0007$) for polyunsaturated fatty acids and vegetable fat, respectively.

We further adjusted for dietary magnesium and cereal fiber (Table 2), both of which were inversely related to diabetes incidence in this population (20). After this adjustment, animal fat was no longer related to diabetes risk ($P = 0.24$). Also attenuated were relations with dietary cholesterol and the Keys score. Comparing the highest to the lowest category of intake, the RRs for cholesterol and the Keys score were 1.17 (95% CI 1.01–1.37) and 1.17 (0.99–1.38), respectively.

After simultaneous adjustment for other dietary fat subtypes, vegetable fat remained clearly associated with type 2 diabetes (Table 3). Relative risks across quintiles of vegetable fat intake were 1.00, 0.90, 0.87, 0.84, and 0.82 ($P = 0.02$), and they did not change appreciably after further adjustment for vitamin E. When we substituted polyunsaturated fatty acids for saturated fatty acids and vegetable fat for animal fat, we found that they were inversely related to diabetes risk. RRs across polyunsaturated fat intake were 1.0, 0.92, 0.89, 0.83, and 0.84 ($P = 0.02$). Comparing the highest to the lowest category of vegetable intake, the RR was 0.78 (95% CI 0.67–0.91).

We analyzed foods and food groups that contribute to fat intake. Total meat intake was positively correlated to diabetes risk. Relative risks across categories of intake were 1.0, 1.04, 1.07, 1.15, and 1.35 ($P = 0.0004$). After adjustment for vegetable fat and dietary cholesterol, magnesium, and cereal fiber, the relative risks across categories of intake were 1.0, 1.01, 1.02, 1.06, and 1.19 ($P = 0.07$). Eggs were also positively correlated to diabetes risk, with RRs across categories of intake of 1.0, 1.01, 1.11, and 1.21 ($P = 0.02$). This relation was eliminated after the adjustment for dietary cholesterol ($P = 0.99$). Foods high in vegetable fat, such as nuts, olive oil dressing, and margarine were not clearly related to diabetes risk.

There was no evidence in these data for modification of the relation between vegetable fat and diabetes by BMI. The relative risks for increasing vegetable fat intake roughly indicated a 20% reduction in diabetes when comparing the highest and lowest tertiles of vegetable fat in both the lowest and highest BMI tertiles. Similarly, we did not find support for effect modification by physical activity, alcohol consumption, or vitamin E intake.

CONCLUSIONS — Data from this prospective study of older women indicate that the composition of dietary fat may play a role in the development of type 2 diabetes. After adjusting for potential confounding variables and animal fat, we found an inverse relation between vegetable fat and incident type 2 diabetes. Polyunsaturated fatty acid was inversely related to diabetes risk when substituted for saturated fatty acid, and the Keys dietary score was positively correlated to diabetes.

There was no relation between dietary fat and diabetes in several prospective studies, which was consistent with our findings (5–8). Among 1,462 Swedish women, the mean intake of total dietary fat (based on a diet history) did not differ for women who did and did not go on to develop diabetes (7). The percent of energy derived from fat did not differ among Pima Indian women who developed diabetes compared with those who remained disease free (6).

However, other prospective studies have shown a positive correlation between diabetes and total dietary fat. Among subjects with impaired glucose tolerance (IGT), total dietary fat (assessed by 24-h recall) predicted conversion to diabetes within 1–3 years (21). In two cohorts of the Seven Countries Study, the percent of energy from fat predicted diabetes and was positively correlated to postload glucose levels after 20 years of follow-up (22).

Results from cross-sectional studies have been similarly mixed. A positive correlation between insulin sensitivity, derived from postload insulin and glucose measurements, and total dietary fat, which was reported by Lovejoy and DiGi-

Table 2—*Multivariate-adjusted relative risks of incident diabetes across quintiles of dietary fat variables among 35,988 Iowa women, 1986–1992*

Variable	Quintile of Intake 1	2	3	4	5	P for trend
Total dietary fat						
Median intake (g/day)	55.7	56.1	60.1	66.8	86.6	
Cases	332	351	380	387	440	
Relative risk* (95% CI)	1.00	1.04 (0.88–1.21)	1.01 (0.86–1.18)	1.02 (0.87–1.19)	1.04 (0.89–1.21)	0.69
Relative risk† (95% CI)	1.00	1.00 (0.85–1.17)	0.95 (0.81–1.11)	0.93 (0.79–1.10)	0.89 (0.75–1.05)	0.11
Saturated fatty acids						
Median intake (g/day)	19.3	19.2	20.4	23.2	31.8	
Cases	313	342	386	432	417	
Relative risk* (95% CI)	1.00	1.07 (0.91–1.26)	1.10 (0.94–1.30)	1.16 (1.00–1.36)	1.11 (0.95–1.29)	0.14
Relative risk† (95% CI)	1.00	1.05 (0.89–1.24)	1.06 (0.90–1.25)	1.10 (0.94–1.29)	1.00 (0.85–1.18)	0.91
Polyunsaturated fatty acids						
Median intake (g/day)	8.9	9.2	10.4	12.2	16.6	
Cases	412	372	369	351	386	
Relative risk* (95% CI)	1.00	0.93 (0.80–1.08)	0.90 (0.78–1.05)	0.84 (0.73–0.98)	0.87 (0.75–1.00)	0.03
Relative risk† (95% CI)	1.00	0.94 (0.81–1.08)	0.91 (0.78–1.06)	0.85 (0.73–0.99)	0.88 (0.76–1.02)	0.05
Monounsaturated fatty acids						
Median intake (g/day)	20.4	20.9	22.7	25.7	33.8	
Cases	336	354	378	368	454	
Relative risk* (95% CI)	1.00	1.01 (0.86–1.18)	1.05 (0.90–1.23)	0.95 (0.81–1.11)	1.06 (0.91–1.23)	0.58
Relative risk† (95% CI)	1.00	0.99 (0.84–1.16)	1.01 (0.86–1.19)	0.90 (0.76–1.06)	0.96 (0.82–1.13)	0.48
Long-chain ω-3 fatty acids						
Median intake (g/day)	0.03	0.09	0.13	0.20	0.39	
Cases	387	360	358	345	440	
Relative risk* (95% CI)	1.00	0.97 (0.83–1.12)	0.99 (0.85–1.16)	0.97 (0.83–1.13)	1.15 (1.00–1.33)	0.02
Relative risk† (95% CI)	1.00	0.98 (0.84–1.14)	1.01 (0.87–1.18)	0.99 (0.85–1.15)	1.20 (1.03–1.39)	0.006
Trans fatty acids						
Median intake	2.2	2.4	2.8	3.5	5.2	
Cases	363	388	379	360	400	
Relative risk* (95% CI)	1.00	1.01 (0.87–1.18)	0.93 (0.80–1.09)	0.86 (0.74–1.01)	0.88 (0.76–1.03)	0.03
Relative risk† (95% CI)	1.00	0.99 (0.85–1.15)	0.90 (0.77–1.05)	0.82 (0.70–0.97)	0.83 (0.70–0.97)	0.004
Cholesterol						
Median intake (mg/day)	185	201	237	281	382	
Cases	325	301	368	402	494	
Relative risk* (95% CI)	1.00	0.87 (0.74–1.03)	1.07 (0.91–1.25)	1.10 (0.94–1.28)	1.24 (1.07–1.43)	0.0001
Relative risk† (95% CI)	1.00	0.86 (0.73–1.01)	1.04 (0.89–1.22)	1.06 (0.91–1.24)	1.17 (1.01–1.37)	0.002
Keys score						
Median	31.4	37.4	41.6	46.0	53.2	
Cases	283	346	400	422	439	
Relative risk* (95% CI)	1.00	1.14 (0.97–1.34)	1.21 (1.03–1.42)	1.25 (1.07–1.46)	1.27 (1.08–1.49)	0.002
Relative risk† (95% CI)	1.00	1.12 (0.95–1.32)	1.17 (1.00–1.37)	1.19 (1.01–1.39)	1.17 (0.99–1.38)	0.06
Animal fat						
Median intake (g/day)	29.1	29.8	33.7	40.4	56.8	
Cases	317	344	364	416	449	
Relative risk* (95% CI)	1.00	1.08 (0.92–1.27)	1.08 (0.92–1.27)	1.17 (1.01–1.37)	1.19 (1.02–1.39)	0.01
Relative risk† (95% CI)	1.00	1.06 (0.90–1.25)	1.04 (0.89–1.23)	1.12 (0.95–1.31)	1.09 (0.93–1.28)	0.24
Vegetable fat						
Median intake (g/day)	18.6	20.2	23.7	29.2	41.7	
Cases	434	377	358	349	372	
Relative risk* (95% CI)	1.00	0.88 (0.76–1.02)	0.84 (0.73–0.98)	0.81 (0.69–0.94)	0.78 (0.68–0.91)	0.0007
Relative risk† (95% CI)	1.00	0.86 (0.76–1.03)	0.85 (0.73–0.99)	0.81 (0.70–0.95)	0.79 (0.68–0.92)	0.001

*Proportional hazards regression models were adjusted for age, total energy, WHR (quintiles: <0.762, 0.763–0.805, 0.806–0.848, 0.849–0.901, >0.901), BMI (quintiles: <22.7, 22.7–24.8, 24.9–27.0, 27.1–30.2, >30.2), physical activity (four levels each for frequency of vigorous and moderate activity: never or rarely, a few times a year, from a few times a month to about once a week, or 2 times a week or more), cigarette smoking (none, 1–19 pack-years, 20–39 pack-years or ≥40 pack-years), alcohol consumption (none, <4 g per day, from 4–10 g per day, or ≥10 g per day), education (no high school diploma, high school diploma, college or vocational school but no degree, or college degree), marital status (currently married, never married, separated or divorced, or widowed), residential area (farm, rural or small town with population up to 2,499, town of population from 2,500–10,000 or city or town with population >10,000), and hormone replacement therapy (current, former, or never). †Additionally adjusted for energy-adjusted dietary magnesium (quintiles) and cereal fiber (quintiles).

Table 3—*Multivariate-adjusted* relative risks of incident diabetes across quintiles of dietary fat variables among 35,988 Iowa women, 1986–1992*

Variable	Quintile of Intake					*P* for trend
	1	2	3	4	5	
Saturated fatty acids						
Relative risk† (95% CI)	1.00	1.06 (0.89–1.27)	1.07 (0.89–1.29)	1.11 (0.91–1.36)	0.95 (0.76–1.19)	0.71
Polyunsaturated fatty acids						
Relative risk† (95% CI)	1.00	0.94 (0.81–1.09)	0.93 (0.79–1.08)	0.88 (0.74–1.03)	0.90 (0.75–1.07)	0.19
Relative risk‡ (95% CI)	1.00	0.92 (0.79–1.07)	0.89 (0.76–1.04)	0.83 (0.71–0.98)	0.84 (0.71–0.98)	0.02
Monounsaturated fatty acids						
Relative risk† (95% CI)	1.00	0.99 (0.83–1.19)	1.03 (0.84–1.27)	0.93 (0.74–1.18)	1.02 (0.78–1.34)	0.93
Long-chain ω-3 fatty acids						
Relative risk† (95% CI)	1.00	0.97 (0.83–1.12)	0.99 (0.84–1.15)	0.94 (0.80–1.10)	1.11 (0.94–1.30)	0.14
Trans fatty acids						
Relative risk† (95% CI)	1.00	1.01 (0.86–1.19)	0.94 (0.79–1.12)	0.88 (0.73–1.06)	0.92 (0.75–1.11)	0.20
Cholesterol						
Relative risk† (95% CI)	1.00	0.84 (0.71–1.00)	1.02 (0.86–1.22)	1.03 (0.87–1.23)	1.11 (0.92–1.33)	0.07
Animal fat						
Relative risk§ (95% CI)	1.00	1.04 (0.88–1.22)	0.97 (0.82–1.15)	0.99 (0.83–1.18)	0.89 (0.73–1.07)	0.18
Vegetable fat						
Relative risk§ (95% CI)	1.00	0.90 (0.78–1.04)	0.87 (0.75–1.01)	0.84 (0.72–0.98)	0.82 (0.70–0.97)	0.02
Relative risk‖ (95% CI)	1.00	0.88 (0.76–1.02)	0.84 (0.72–0.97)	0.81 (0.6–0.94)	0.78 (0.67–0.91)	0.001

*All models included variables listed in Table 2, reference 2 and dietary protein (quintiles); †model included saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, *trans* fatty acids, ω-3 fatty acids, and cholesterol (quintiles); ‡model included total fat, monounsaturated fatty acids, *trans* fatty acids, and ω-3 fatty acids quintiles; §model included vegetable fat and animal fat (quintiles); ‖model included total fat (quintiles).

rolamo (23), was apparent only before adjusting for BMI. Two other studies also found no association with fasting insulin after accounting for BMI (24,25), whereas another study (26) found that total fat was unrelated to fasting insulin in univariate and multivariate analyses. In contrast, several studies (27–30) found a positive correlation between total dietary fat and fasting or postprandial insulin (independent of BMI) or a negative correlation with insulin sensitivity (34).

Our findings are consistent with some, but not all, prospective epidemiological studies that have examined subtypes of dietary fatty acids. In one study, vegetable fat and polyunsaturated fatty acids were inversely related to incident type 2 diabetes among lean women but not among obese women (31); saturated fatty acids, monounsaturated fatty acids, and animal fats were not related to diabetes. Although statistically nonsignificant, the RRs (0.85 among women and 0.83 among men comparing the highest with the lowest quintile of vegetable fat consumption) for incident diabetes reported in two studies by Salmeron and colleagues (32,33) were strikingly similar to ours for vegetable fat. Incident diabetes and conversion to diabetes were positively correlated to saturated fat and unrelated to polyunsaturated fat in two other follow-up studies (21,22). These findings are more in line with those from most cross-sectional studies, in which saturated fat was positively correlated to fasting insulin (24,26–29) or area under the insulin curve (34) and inversely related to insulin sensitivity (34). The findings for polyunsaturated fatty acids from these studies were more varied, showing inverse (34), positive (27), and no correlation (25,28,29,34) with insulin concentrations or sensitivity.

The inverse relationship between diabetes and vegetable fat remained after adjustment for other dietary fat, but that for polyunsaturated fatty acids did not. Likewise, Salmeron and colleagues (32,33) reported no association between polyunsaturated fatty acids and diabetes. When the highest to the lowest quintiles of polyunsaturated fatty acid intake were compared, the adjusted relative risks for diabetes were 0.97 in women and 1.01 in men. Vegetable fat includes fats found in nonanimal sources, including fruits and vegetables, grains, nuts, and oils. It is possible that vegetable fat represents the combination of several potentially healthful fat subtypes, including polyunsaturated fatty acids and monounsaturated fatty acids, from vegetable sources. Furthermore, although we considered several dietary factors that have been hypothesized to relate to diabetes risk, such as vitamin E, cereal fiber, and magnesium, vegetable fat will be highly correlated with any number of additional nutrients that we did not include, which may influence diabetes risk.

Although polyunsaturated fatty acids were not related to diabetes after adjusting for all other fatty acids, a true inverse relation is possible. It may be argued that dietary factors are so highly correlated that a high degree of attenuation is inevitable with simultaneous adjustment for dietary factors. When substituted for saturated fatty acids in the diet, polyunsaturated fatty acids were inversely related to diabetes, whereas the Keys score was positively correlated to diabetes. These findings are consistent with data from a 10-year follow-up study of middle-aged men who did and did not develop type 2 diabetes (35). Men who did develop type 2 diabetes had a higher proportion of saturated fatty acids and a lower proportion of linoleic acid in serum cholesterol esters, which in part reflects dietary fatty acid composition, than men who did not de-

velop type 2 diabetes (35). A cross-sectional study of 45 subjects found no relationship between insulin sensitivity and the ratio of dietary polyunsaturated fatty acids to saturated fatty acids (23).

Feeding studies support a positive correlation between monounsaturated fatty acid intake and insulin sensitivity (36–39). In our study population, monounsaturated fatty acid consumption was more highly correlated with saturated and animal fat consumption (correlation coefficients 0.70 and 0.62, respectively) than with polyunsaturated and vegetable fat consumption (correlation coefficients 0.43 and 0.29, respectively). This may limit our ability to isolate the effects of individual fats; it underscores the importance of the specific dietary characteristics of studied populations. Such correlations will be somewhat population-specific; they may have contributed to the positive associations between monounsaturated fat and incident diabetes (22), to the progression to diabetes from IGT (21), and to fasting insulin (27,28) in some studies, but the inverse relation with insulin sensitivity (34) appears in other studies.

In our study, diabetes was positively correlated to dietary cholesterol and the Keys score. Similarly, Feskens et al. (22) found a positive correlation between dietary cholesterol and 20-year diabetes incidence in two Seven Countries Study cohorts. Conversely, two cross-sectional studies found no association between dietary cholesterol and fasting or postprandial insulin (26,34). It is unclear how dietary cholesterol may affect diabetes incidence, and our findings should be interpreted with caution, particularly because the association was attenuated after adjustment for other dietary fat variables.

Dietary cholesterol is related to serum cholesterol levels to some extent (40), and it is possible that changes in serum cholesterol explain the association between dietary cholesterol and diabetes risk (40). Our findings for the Keys score are consistent with the finding that the Keys dietary score indicates positive changes in serum cholesterol predicted by positive changes in dietary cholesterol and saturated fatty acids and negative changes in polyunsaturated fatty acids (13). In two other studies, the univariate or age-adjusted rate of incident diabetes was 50% greater in the highest than in the lowest category of serum cholesterol, although the estimate from only one study was statistically significant (5,8). However, it is difficult to distinguish the relations between the Keys score and serum cholesterol from the relation between the Keys score and saturated and polyunsaturated fatty acid intake, for which the evidence of an association with diabetes is far more compelling.

Differential intakes of dietary fat subtypes may affect diabetes risk by modifying the fatty acid composition of the phospholipid membrane, which may play a role in blood glucose regulation through effects on insulin secretion, insulin receptor properties, and glucose transport. Borkman et al. (41) reported significant inverse relations between fasting serum insulin and the content of ω-3 and ω-6 fatty acids within skeletal-muscle phospholipids. Compared with saturated fatty acids, polyunsaturated fatty acids appear to enhance insulin secretion (42), and saturated fatty acids have been shown to decrease insulin binding to receptors and to impair glucose transport (9).

Error in the measurement of diet, diabetes, and covariates in this study may have limited our ability to obtain accurate relative-risk estimates. Random measurement error in dietary exposures most frequently attenuates risk estimates (43). Of particular interest is the potential for misclassification of *trans* fatty acid intake that resulted from temporal changes in the consumption and manufacturing patterns of *trans* fatty acids in the U.S. during the 1980s, when many consumers switched to soft, reduced *trans* fat margarine and industry ceased the partial hydrogenation of household salad and cooking oils (44). These changes could have resulted in a large misclassification of *trans* fatty acid intake in our population.

The validation study correlation coefficient for cholesterol was quite low (0.21). This did not prevent us from detecting an association between cholesterol and diabetes, but it may reflect a degree of random error in this variable that prevented us from detecting a truly larger magnitude of association. Our validation sample was quite small ($n = 44$), and it is possible that our sample was aberrant; correlations for other fat variables were similar to those obtained in another large study of women, in which the correlation coefficient for cholesterol was 0.61 (14).

Incident cases of diabetes were ascertained by self-report. Our validation study (see above) suggested that participants over-reported diabetes compared with physician diagnoses (16). This is consistent with findings from one study (45) in which 29 of 44 (66%) positive reports of diabetes were validated with medical records. Nonvalidated positive reports may nonetheless reflect some level of diabetes. One study found that several persons with nonvalidated positive reports of diabetes had some history of glycosuria (46). Thus, nondiabetic concentrations of blood glucose may not be entirely benign, and women who falsely reported a diagnosis of diabetes may still have some level of underlying disease, such as IGT. This possibility is supported by the change in the diagnostic criteria for diabetes to lower levels of fasting glucose (47). The ascertainment of diabetes in the present study was sensitive enough to confirm associations with other risk factors for diabetes (i.e., weight [16], physical activity [48], and dietary fiber [20]) obtained from studies with validated diabetes. Assuming that the error in diabetes ascertainment was independent and nondifferential, present findings would only be strengthened by more accurate ascertainment of disease.

Dietary fat may contribute to the etiology of type 2 diabetes. After adjusting for nondietary and dietary covariates, we found that vegetable fat was inversely related to incident diabetes in this population of older Iowa women. In addition, substituting polyunsaturated fatty acids for saturated fatty acids appeared to reduce the rate of diabetes.

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Symposium: Dairy Product Components and Weight Regulation

The Conjugated Linoleic Acid (CLA) Isomer, t10c12-CLA, Is Inversely Associated with Changes in Body Weight and Serum Leptin in Subjects with Type 2 Diabetes Mellitus[1,2]

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ABSTRACT Isomers of conjugated linoleic acid (CLA) are found in beef, lamb and dairy products. Diets containing CLA reduce adipose mass in various depots of experimental animals. In addition, CLA delays the onset of diabetes in the ZDF rat model for obesity-linked type 2 diabetes mellitus. We hypothesize that there would be an inverse association of CLA with body weight and serum leptin in subjects with type 2 diabetes mellitus. In this double-blind study, subjects with type 2 diabetes mellitus were randomized into one of two groups receiving either a supplement containing mixed CLA isomers (CLA-mix; 8.0 g daily, 76% pure CLA; $n = 12$) or a supplement containing safflower oil (placebo; 8.0 g daily safflower oil, $n = 9$) for 8 wk. The isomers of CLA in the CLA-mix supplement were primarily c9t11-CLA (~37%) and t10c12-CLA (~39%) in free fatty acid form. Plasma levels of CLA were inversely associated with body weight ($P < 0.05$) and serum leptin levels ($P < 0.05$). When levels of plasma t10c12-CLA isomer were correlated with changes in body weight or serum leptin, t10c12-CLA, but not c9t11-CLA, was inversely associated with body weights ($P < 0.05$) and serum leptin ($P < 0.02$). These findings strongly suggest that the t10c12-CLA isomer may be the bioactive isomer of CLA to influence the body weight changes observed in subjects with type 2 diabetes. Future studies are needed to determine a causal relationship, if any, of t10c12-CLA or c9t11-CLA to modulate body weight and composition in subjects with type 2 diabetes. Furthermore, determining the ability of CLA isomers to influence glucose and lipid metabolism as well as markers of insulin sensitivity is imperative to understanding the role of CLA to aid in the management of type 2 diabetes and other related conditions of insulin resistance. J. Nutr. 133: 257S–260S, 2003.

KEY WORDS: • *conjugated linoleic acid* • *body weight* • *leptin* • *type 2 diabetes mellitus*

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids (PUFA) that exist as positional and stereoisomers of conjugated dienoic octadecadienoate (18:2). The predominant isomer in foods is the c9t11-CLA isomer (1,2) (also called "rumenic acid") (3) followed by 7,9-CLA (c/t), 11,13-CLA (c/t), 8,10-CLA (c/t) then t10c12-CLA isomer (1). CLA is found primarily in foods such as beef, lamb and dairy foods (2,4,5). A synthetic mixture of CLA (referred to as CLA-mix) may also be found in nutritional supplements and is composed primarily of the c9t11-CLA and the t10c12-CLA isomers (Fig. 1).

Conjugated linoleic acid alters adipose tissue distribution

The conjugated fatty acids, or CLA, reduce adiposity in normoglycemic (nondiabetic) individuals as well as experimental animals including mice, rats, and pigs (6–8). Total adipose tissue mass was reduced by over 50% in mice fed a diet containing CLA-mix (1.0 wt %) compared to mice fed a control diet (without CLA). The reduction of adiposity by dietary CLA could be sustained in mice even after CLA was removed from the diet (9). Subsequent studies in nonobese mice demonstrated that some depots of fat mass were more sensitive than others to the effects of CLA. Diets with CLA-mix were especially effective in reducing adipose tissue mass in retroperitoneal and epididymal white adipose tissues (10–12) as well as brown adipose tissue (11).

There appears to be an isomer-specific effect of CLA on adiposity. t10c12-CLA is much more effective at lowering adipose tissue mass than the c9t11-CLA isomer in mice (13). In addition, t10c12-CLA is the effective isomer for modulating gene expression in cultured 3T3-L1 preadipocytes (14). The ability of CLA to reduce adipose tissue mass occurs

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[2] Supported by Pharmanutrients, Inc. (Lake Bluff, IL) and Natural, Inc. (Chicago, IL).

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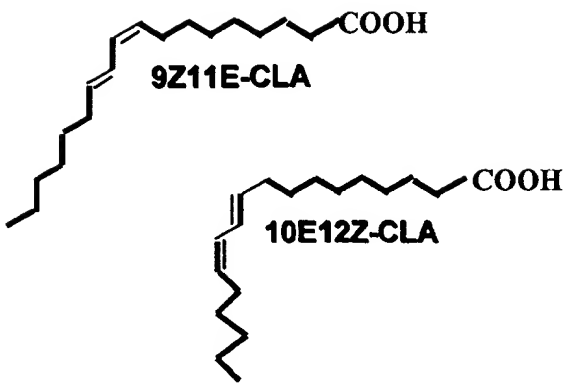

FIGURE 1 Structures of c9t11-CLA and t10c12-CLA.

regardless of food intake or fat level (total fat level, 6.5–20.0 wt %) in growing mice so that feed efficiency is affected (6,9). Furthermore, CLA reduces leptin in diabetic (ZDF) rats (15), nondiabetic mice (11) and humans with type 2 diabetes (Belury, M. A., unpublished data). Because leptin is a hormone secreted by adipose tissue that regulates food intake, it may be of significance to note that dietary CLA reduces food intake in mice and rats (7,16). However, supplementation with CLA in nonobese humans (3.0 g/d) has a modest and transient effect on leptin and had no effect on food intake (17). There is a possibility that a higher dose of CLA and/or longer duration of supplementation of CLA may affect food intake but this is yet to be determined.

Although most studies using nonobese or growing animal models have shown that dietary CLA lowers adipose tissue mass, not all studies show such an inverse relationship between dietary CLA and adipose tissue mass. Obese Zucker rats (8), but not diabetic fatty Zucker (ZDF) rats (12), exhibit an adipose-enhancing effect of dietary CLA-mix (8). In C57BL/6J mice, a mouse model for obesity and insulin resistance, long-term feeding with CLA-mix (1.0% CLA for 8 mo) leads to the formation of lipodystrophy, resulting in complete ablation of brown adipose tissue, increased fat accumulation in the liver and reduced leptin. Eventually, the lipodystrophic mice fed CLA developed insulin resistance (11). However, others have found this effect was transient (18). Mice (C57BLK $Lepr^{dbdb}$/$lepr^{dbdb}$) fed for 12 wk with CLA-mix diets exhibited induction of insulin resistance after 5 wk of feeding but a restoration of insulin sensitivity by 11 wk (18). These data suggest the effect of CLA on adiposity may be dependent on preexisting adiposity and/or insulin sensitivity.

In adult humans, the association of supplementation with CLA-mix and reductions of body weight or adipose tissue mass has been demonstrated in some (19–21) but not all (22,23) studies. In one study, overweight or obese human subjects supplemented with CLA-mix (3.4–6.0 g/d) for 12 wk exhibited a significant reduction of fat mass (20), whereas another study showed no such benefit of CLA supplementation (23). More recent studies have demonstrated that CLA supplementation reduces body weight, leptin and/or body adiposity in people (19,21; Belury, M. A., unpublished data). It is likely that dose, duration (short- vs. long-term) and the isomeric composition of CLA will each impact the ability of CLA to affect obesity in humans. In addition, how strain-, species-, age- and sex-specific effects of various isomers of CLA to influence adipose tissue accumulation, either in obese humans or those seeking to prevent adipose gain, is yet to be determined. Furthermore, a well-controlled study to determine the role of CLA in altering the distribution of adipose tissue (e.g., intraabdominal vs. subcutaneous fat) using validated methods has yet to be reported.

Conjugated linoleic acid affects body weight in human subjects with type 2 diabetes

Several risk factors for developing type 2 diabetes have been identified, including obesity, impaired glucose tolerance, some ethnicities (e.g., African-American, Asian, Pacific Islanders and Native American), advancing age, gestational diabetes, a positive family history of type 2 diabetes and lipid abnormalities. Central to all of these risk factors is the influence of obesity. In fact, lifestyle intervention resulting in a modest reduction of body weight (~7%) was associated with a 58% reduction in the incidence of diabetes in a cohort of people who were considered at high risk for developing this disease (24).

Previous studies from our laboratory demonstrated that CLA delays the onset of diabetes in the ZDF rat model. Therefore, we designed a study to elucidate the relationship of CLA to improvements in the management of type 2 diabetes mellitus in humans (Belury, M. A., unpublished results). Criteria for enrollment in this study included the requirement that subjects were not currently using medication for glucose control. The study was double blinded, where subjects were randomized in a block design according to fasting blood glucose values for either CLA supplementation (n = 11; 6.0 g/d) or safflower placebo supplementation (n = 10) for a duration of 8 wk. The CLA-mix supplement was composed of c9t11-CLA (~37%), t10c12-CLA (~39%), palmitic (6%), stearic (4%), oleic and linoleic (15%) acids in free fatty acid form.

Dietary intake of energy and fat quantity and quality were measured by use of a 3-d diet record followed by four repeated measures using a 24-h recall analysis. Dietary records were analyzed with the Minnesota Database (University of Minnesota, St. Paul, MN). Dietary intake of energy (kcal), fat (% kcal) or fat quality were similar between treatment groups at baseline. Subjects were instructed to maintain a healthy diet using the Food Guide Pyramid as a guide and were asked not to change their diet or activity habits for the 8-wk intervention period. There was no significant change in dietary energy or fat calories between week 0 and week 8 for either group. Compliance of subjects for pill consumption was reported to be >80–100% for pills consumed for all subjects in either group. Through use of a plasma biomarker for compliance, the accumulation of the t10c12-CLA isomer in plasma was significant ($P < 0.05$) for subjects supplemented with CLA (data not shown). In addition to measuring body weight and dietary composition, serum leptin was measured by radioimmunoassay (LINCO, St. Charles, MO). By week 8, supplementation with CLA (6.0 g CLA/d) was associated with decreases in fasting plasma glucose in nine out of 11 (81%) subjects on CLA supplementation and two out of 10 (20%) subjects on safflower supplementation.

When the level of CLA that accumulated in plasma was correlated with the change in body weight, there was a significant inverse correlation ($r = -0.4234$; $P < 0.05$) (**Fig. 2**). In addition, the plasma level of CLA was significantly inversely correlated with serum leptin ($r = -0.4275$; $P < 0.05$). Because the c9t11-CLA isomer is the predominant isomer found in foods such as beef, lamb and dairy foods, we determined the association of this isomer in plasma to changes in body weight or serum leptin. Associations of plasma c9t11-CLA to body weight or serum leptin were not significant ($r = -0.2873$ and $r = -0.3224$, respectively; data not shown). Because the

t10c12-CLA isomer has been shown to be the bioactive isomer to reduce adipose tissue in experimental animals, we determined the correlation coefficient of changes in body weight and leptin vs. t10c12-CLA levels in plasma.

In contrast to findings with the c9t11-CLA levels in plasma, the correlation coefficients for the t10c12-CLA isomer vs. changes in body weight or serum leptin were significant (body weight, $r = -0.4309$; $P < 0.05$; leptin, $r = -0.5260$, $P < 0.02$) (Fig. 3). Furthermore, the coefficients were stronger than the relationship for total plasma CLA to either body weight or serum leptin. These data suggest the lower body weights and serum leptin values in the subjects supplemented with CLA are attributed to the accumulation of the t10c12-CLA isomer in the plasma.

Unfortunately, body fat mass and distribution were not measured in this study. However, a recent study suggests a lowering of abdominal adiposity where there was an inverse relationship between supplementation with CLA-mix (4.2 g/d) for 12 wk and sagittal abdominal diameter (25) in overweight subjects. It is possible that the changes in serum leptin values that we observed may simply reflect a reduction in adipose tissue mass; however, leptin secretion may be more highly associated with reduced subcutaneous, not intraabdominal, adipose tissue mass (26). Of further note, a second neuroendocrine hormone, adiponectin, may be highly and inversely correlated to intraabdominal adipose mass (27). Unfortunately, we did not measure abdominal fat mass or adiponectin in this study, although future studies are warranted with such an analysis.

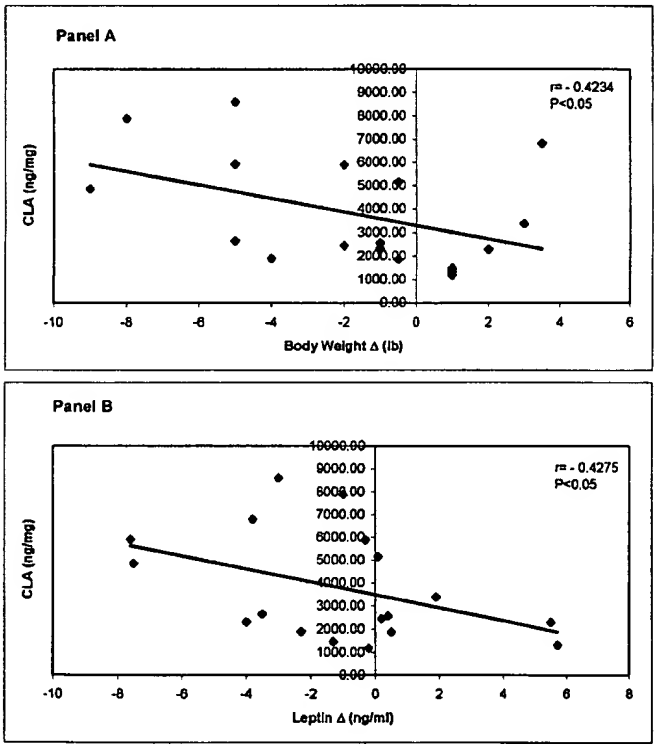

FIGURE 2 Plasma CLA is inversely correlated with (*A*) changes in body weight and (*B*) changes in serum leptin in subjects with type 2 diabetes. Subjects were supplemented with CLA or safflower capsules (8.0 g/d) for 8 wk. The level of CLA was determined by high performance liquid chromatography and gas chromatography as described previously (28,29).

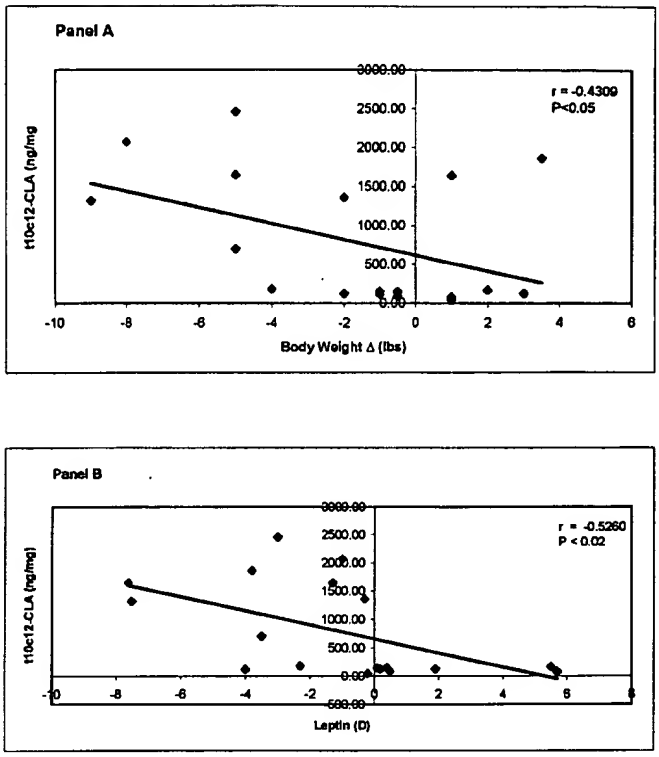

FIGURE 3 Plasma t10c12-CLA is inversely correlated with (*A*) changes in body weight and (*B*) changes in serum leptin in subjects with type 2 diabetes. Methods are as described in Figure 2.

Summary

The intake of dairy foods has been shown to be correlated with reduced body fat and enhanced insulin sensitivity in various cohorts. A potential group of bioactive compounds that could explain these effects might be CLA. However, our data suggest that there is a stronger correlative of the t10c12-CLA isomer than the naturally occurring rumenic acid (c9t11-CLA). Further work is needed to address the specific actions of the t10c12-CLA vs. c9t11-CLA isomers in the management of body weight in subjects with type 2 diabetes. In addition to determining the influence of CLA to reduce intraabdominal adiposity, it is important to determine the extent that favorable modifications of adipose tissue (e.g., reduction and/or redistribution) by CLA or various CLA isomers may affect glucose and lipid metabolism as well as insulin sensitivity in subjects with type 2 diabetes mellitus.

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Inhibition of Carcinogenesis by Conjugated Linoleic Acid: Potential Mechanisms of Action[1]

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ABSTRACT Conjugated linoleic acid (CLA) is composed of positional and stereoisomers of octadecadienoate (18:2); it is found in foods derived from ruminants (beef and lamb as well as dairy products from these sources). When a mixture of isomers is fed to experimental animals, chemically induced tumorigenesis of mammary, skin and colon is reduced. Importantly, many isomers of CLA are readily metabolized to desaturated/elongated products as well as β-oxidized products, suggesting that these metabolites may be important anticancer compounds. Mechanisms of inhibition of carcinogenesis may include reduction of cell proliferation, alterations in the components of the cell cycle and induction of apoptosis. In addition, CLA modulates markers of immunity and eicosanoid formation in numerous species as well as lipid metabolism and gene expression. It is likely that CLA exerts inhibitory properties in carcinogenesis via one or more of these pathways with some tissue specificity. This review will explore recent advances in putative mechanisms of reduction of carcinogenesis by CLA. J. Nutr. 132: 2995–2998, 2002.

KEY WORDS: • conjugated linoleic acid • carcinogenesis • CLA isomers • anticarcinogenic

Conjugated linoleic acid (CLA)[3] refers to a group of polyunsaturated fatty acids that exist as positional and stereoisomers of octadecadienoate (18:2). CLA is found in foods such as beef and lamb as well as dairy foods derived from these ruminant sources (1,2). The double bonds of CLA may be in the positions of 7,9; 8,10; 9,11; 10,12; or 11,13 and the 3-dimensional geometric combinations of *cis* and/or *trans* configurations. The major isomers in foods are in the following rank order: *c*9*t*11-CLA (also called rumenic acid) > *t*7*c*9-CLA > 11,13-CLA (*c*/*t*) > 8,10-CLA (*c*/*t*) > *t*10*c*12-CLA isomer > other isomers (2–4). Importantly, the majority of experiments performed in experimental animals have used a synthetic mixture of CLA isomers containing primarily *c*9*t*11-CLA and *t*10*c*12-CLA (**Fig. 1**) [reviewed in 5)].

Numerous physiologic properties have been attributed to CLA including action as an anticarcinogenic, antiatherosclerotic, antiadipogenic and antidiabetogenic agent [reviewed in 5–7)]. In addition, CLA modulates immunity and thrombosis as well as fatty acid biochemistry, lipid metabolism and gene expression in the liver, muscle and adipose tissues (6). There are several recent reviews on the effects and mechanisms of CLA in biological systems, including cancer (7). Therefore, this review will focus on recently identified mechanisms by which CLA inhibits carcinogenesis.

Dietary CLA inhibits carcinogenesis in experimental animals

As a component of semipurified diets, CLA inhibits cancer in several animal models. In particular, CLA inhibits dimethylbenz(a)anthracene-induced tumorigenesis of skin, mammary and forestomach neoplasia (8–10). In addition, when a synthetic mixture of CLA isomers (~45% *c*9*t*11-CLA, ~42% *t*10*c*12-CLA with several other remaining isomers comprising minor amounts) is provided in diets (0.5–1.5 g/100 g) either during or after initiation, chemically induced skin tumor promotion or mammary and colon tumorigenesis are inhibited (10–13). Furthermore, CLA inhibits the growth of transplanted cell lines derived from mammary (14) and prostate (15) cancers. Although the role of CLA in inhibiting carcinogenesis is convincing, not all studies have shown inhibition. In fact, CLA did not alter the growth of transplanted prostate (7) and breast (16) cancer cells and did not reduce tumorigenesis in an intestinal model of colon carcinogenesis using the Apc^{Min} mouse (17). No studies have yet reported that CLA enhances tumorigenesis.

In conjunction with identifying the inhibitory properties of CLA in various tumor models, efforts have been made to elucidate the role of CLA in modulating the stages of carcinogenesis known as initiation, promotion and progression. In particular, the anticarcinogenic property of CLA was first identified during the initiation stage of skin carcinogenesis, a stage associated with a genetic alteration in a subset of cells in the target tissue (8). During initiation, CLA modulates events such as free radical–induced oxidation, carcinogen metabolism and carcinogen-DNA adduct formation in some tissues (7). Findings have been ambiguous. In fact, a recent study in male rats demonstrated tissue-specific effects of CLA on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine–induced mutation frequencies (18): Dietary CLA (0.5 g/100 g) reduced mutation frequency in the distal colon, but had no effect or enhanced mutation frequency in the proximal colon and cecum of rats.

In addition to tissue- and/or tumor model–specific effects of CLA on tumor initiation, several studies demonstrated that CLA inhibits carcinogenesis postinitiation (10,11,13,19,20). In chemically induced mammary carcinogenesis, there may be an optimal time for exposure to CLA, i.e., the inhibitory properties of CLA on chemically induced mammary carcinogenesis were most profound when CLA was fed during mammary gland maturation [between 21 and 42 d of age (19)]. During the promotion stage of skin carcinogenesis, CLA re-

[1] Supported by the American Cancer Society (RPG-CNE87932).
[2] To whom correspondence should be addressed. E-mail: Belury@u.washington.edu.
[3] Abbreviations used: BrdU, bromodeoxyuridine; CLA, conjugated linoleic acid; COX, cyclooxygenase; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptors.

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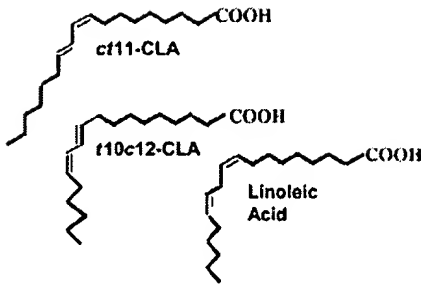

FIGURE 1 Structures of *c9t11*-conjugated linoleic acid (CLA), *t10c12*-CLA and linoleic acid [18:2(n-6)].

duces the yield of mouse skin tumors by a mechanism distinct from its anti-initiator activity (10).

Although a great deal of evidence demonstrates that dietary CLA inhibits the initiation and postinitiation and/or promotion stages of carcinogenesis, its role in the progression stage of carcinogenesis has not been established definitively. Using transplantable tumor models, dietary CLA reduced the growth rates of mammary and prostate cancer cells when implanted in vivo in mice (14,15). In addition, at least one study demonstrated that CLA (0.5–1.0 g/100 g) inhibited the ability of transplanted mammary cancer cells to form secondary tumors in mice (21). Furthermore, the CLA-responsive chemically induced mammary carcinogenesis model (10) is a model for human breast cancer ductal carcinomas in situ. Therefore, data showing that CLA inhibits tumorigenesis in this model are consistent with the possibility that CLA will reduce breast cancer metastasis. However, no studies have addressed the role of CLA in the prevention of metastatic cancer. It is critical to understand how CLA modulates malignant tumor formation and metastasis because the growth of secondary tumors is the major cause of morbidity and mortality in people with cancer.

CLA modulates cell proliferation and apoptosis

In an attempt to identify mechanisms of action, recent efforts have focused on elucidating how CLA modulates events that occur postinitiation and/or during promotion. The promotion stage involves the clonal expansion of initiated cells to form a benign tumor. This stage of carcinogenesis represents a premalignant state in which tumors form as a result of imbalances between dysregulated differentiation, enhanced cell proliferation and/or reduced apoptosis (or programmed cell death). CLA reduces the proliferation of numerous cell types grown in culture [reviewed in 7)]. In vivo, dietary CLA (1.0 g/100 g) reduces proliferation of terminal end bud and lobuloalveolar bud structures, the sites at which tumors form in both rat and human mammary cancers (22). Furthermore, mammary adenocarcinomas induced by PhIP contained significantly fewer proliferating cell nuclear antigen positive cells in rats fed dietary CLA (0.1 g/100 g) compared with rats fed a control diet without CLA (13). Recent work by Ip and colleagues (23) demonstrated that CLA or *c9t11*-CLA–rich butter fat reduces the rate of incorporation of bromodeoxyuridine (BrdU) and the expression of cyclins A and D. These two cyclins regulate the conversion of G1 → S phase of the cell cycle (Fig. 2). In addition, diets with CLA moderately increased levels of p16 and p27 proteins. These data suggest that CLA reduces cell proliferation by blocking DNA synthesis and cell cycle proteins that regulate this process (13,23). In contrast to findings in mammary carcinogenesis, dietary CLA does not reduce cell proliferation in phorbol ester–induced tumor promotion of mouse skin as measured by hyperplasia or ornithine decarboxylase activity, although c-myc mRNA was modestly reduced (24). Furthermore, dietary CLA enhances cell proliferation and/or ornithine decarboxylase in livers of rats and mice (25,26).

As a counterbalance to cell proliferation, apoptosis offers protection against carcinogenesis via programmed cell death (Fig. 2). Dietary CLA induces apoptosis in numerous tissues including mammary gland (27), liver (25), colon (28) and adipose (29) tissues. In terminal end buds of rat mammary tissue initiated with methylnitrosurea, dietary CLA induces apoptosis in a site-specific manner. The ability of CLA to induce apoptosis in terminal end buds and the premalignant lesions known as intraductal proliferation lesions (27) may have implications for development of this epithelial tissue. Induction of apoptosis by CLA was associated with reduction of Bcl-2 protein within the lesions. The Bcl-2 gene family has differential effects on apoptosis; for example, Bcl-2 and Bcl-x_L suppress apoptosis, whereas others, such as Bax and Bak, promote apoptosis. The ability of Bax to induce apoptosis appears to involve a countereffect on Bcl-2. Although CLA reduced Bcl-2, there was only a moderate effect of CLA on induction of Bax protein. Therefore, it appears that CLA may support elevated apoptosis primarily by reducing the suppressor of apoptosis, bcl-2. Because the inhibitory effects of CLA or *c9t11*-CLA on reductions of BrdU incorporation in mammary epithelium were dependent on the proliferative (vs. quiescent) status of mammary epithelial cells (23), effects of CLA on pivotal signaling events regulating both cell proliferation and apoptosis (e.g., cyclin dependent kinases or check point proteins such as p53) warrant further study.

Effects of CLA on phospholipid metabolism and regulation of gene expression

The cellular mechanisms of modulation of carcinogenesis by CLA are numerous and complex. Several studies have shown that diets with CLA are associated with altered phospholipid-associated fatty acid metabolism and eicosanoid for-

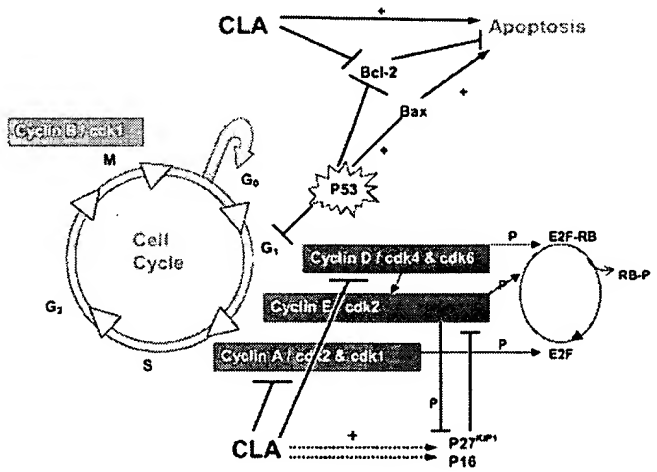

FIGURE 2 Schematic diagram of how conjugated linoleic acid (CLA) may modulate the cell cycle and apoptosis. CLA significantly reduces levels of cyclin A and cyclin D and induces apoptosis in the mammary epithelium (23,27). The tumor suppressor, p53, induces apoptosis and modulates the cell cycle in some cell types under various conditions. Solid lines (—): significant ($P < 0.05$); dotted lines (·····): modest ($P < 0.05$). Abbreviation: cdk, cyclin-dependent kinase.

mation. Eicosanoids modulate tumorigenesis in many tissues including mammary gland, skin, prostate and colon [reviewed in (30)]. Events in carcinogenesis that appear to be particularly sensitive to eicosanoids include cell proliferation, inflammation, local and systemic immunity, platelet aggregation and tissue differentiation. Diets with CLA result in an accumulation of CLA, especially the 9,11-CLA (*c/t*; *t/c*) isomer in phospholipids of tissues [e.g., liver (31), mammary (32), skin (24) and others] and lipid fractions of human sera (M.A. Belury, unpublished data). In addition, when fed as the free fatty acid, dietary CLA alters the relative amounts of numerous other fatty acids in phospholipid fractions (24,28). These findings raise the possibility that CLA, when fed as the free fatty acid, competes with other fatty acids for incorporation into phospholipids and modifies subsequent eicosanoid production (especially from arachidonate, 20:4). In fact, dietary CLA reduces prostaglandin (PG)-E_2 and/or other eicosanoids derived from enzymatic oxidation of arachidonic acid in some tissues (24,28). However, only one study has shown that dietary CLA reduces phospholipid-associated arachidonate (28). In addition, when fed as triglyceride-esterified fatty acid (in CLA-rich butter), CLA does not alter phospholipid associated nonconjugated fatty acids (33). Furthermore, some studies demonstrated that when dietary CLA altered the levels of nonconjugated fatty acids, these changes occurred in neutral lipid fractions of tissues [e.g., adipose (M.A. Belury, unpublished data), skin (24), liver (31) and mammary (32). The relevance of reduced neutral lipid–associated arachidonate levels to altered arachidonate–derived eicosanoids is unclear at present.

Interestingly, when CLA lowers arachidonate-derived eicosanoids such as PGE_2 and $PGF_{2\alpha}$ in colon and skin (24,28), it also reduces tumorigenesis in these tissues. In contrast, at least one study has shown a relationship between an inability of dietary CLA to alter arachidonate-derived eicosanoids with a lack of its inhibition of intestinal tumorigenesis in Min mice (17). Together, these studies indirectly suggest that the mechanism by which CLA inhibits carcinogenesis in some tissues may involve the modulation of arachidonate-derived eicosanoids.

CLA may reduce arachidonate-derived eicosanoids such as prostaglandin-E_2, $PGF_{2\alpha}$, leukotriene-B_4 and leukotriene-C_4 by one of two mechanisms. First, CLA may displace arachidonate incorporation into phospholipids as shown in cultured keratinocytes (34). In addition, dietary CLA displaces the arachidonate precursor, linoleate, in a dose responsive manner in livers of mice fed various doses of CLA (0.5–1.5 g/100 g) in one study (31) but not others (33,35 Belury, M. A. unpublished data). A recent study demonstrated that dietary CLA reduces phospholipid-associated arachidonate in the colonic mucosa of rats (28).

A second explanation for the reduction of arachidonate-derived eicosanoids by CLA may be through inhibition of the constitutive enzyme, cyclooxygenase (COX)-1, and/or the inducible form, COX-2, at the level of mRNA, protein, or activity. CLA or elongated and desaturated products from CLA (e.g., conjugated "arachidonate" or conjugated eicosatetraenoate) may act as antagonists for COX thereby reducing available enzyme (at the level of expression or activity) for arachidonate. Using an in vitro activity assay, CLA or individual isomers inhibited the rate of oxygenation of arachidonate in the presence of COX-1 (36). Furthermore, *c9t11*-CLA and *t10c12*-CLA reduced COX-2 at the levels of mRNA and protein in a cultured macrophage cell line (37).

Although CLA is readily metabolized by Δ^6 desaturase to form numerous downstream products (31–33,38,39), little is known about how CLA modulates metabolism of nonconjugated fatty acids via enzymatic systems such as Δ^6 desaturase-elongase-Δ^5 desaturase. CLA reduces levels of linoleate (18:2) and its desaturated and elongated product, arachidonate (20:4) in mammary tissue (32). In contrast, one study has shown that CLA may modestly enhance levels of neutral lipid–associated arachidonate in the epidermis of mice (24). Furthermore, other studies showed no effect of CLA on arachidonate levels in fat pads (40), liver (33) or small intestine (17). The ability of CLA to alter arachidonate levels may depend on the form of CLA (free fatty acid vs. esterified) as well as tissue- and species-specific effects. The relevance of altered arachidonate levels in neutral lipids vs. phospholipid as a modulator of lipid metabolism and eicosanoid formation is not clear at the present time.

CLA may modulate lipid metabolism in part by a mechanism dependent on the activation of the nuclear hormone receptors, peroxisome proliferator-activated receptors (PPAR) [reviewed in (5)]. In particular, the PPARγ isoform is found in extrahepatic tissues such as adipose, prostate, colon, mammary gland and others. PPARγ2 is a required transcription factor in adipose tissue differentiation [reviewed in (41)]. In addition, thiazolidinediones, high affinity ligands for PPARγ, modulate carcinogenesis in mammary gland, colon and prostate tissues [reviewed in (42)]. Isomers of CLA have moderate affinity for binding to and activating PPARγ (43). Dietary CLA appears to modulate transcription of genes responsive to PPARγ in adipose tissue in vivo [reviewed in (6)] and in vitro (37). Our current attempts to study the ability of CLA to activate PPARγ have focused on downstream metabolites of Δ^6 desaturase metabolism of *c9t11*-CLA or *t10c12*-CLA. In these studies, we have used approaches to block desaturase activity to determine whether reducing metabolites alters activation of PPARγ (43). CV-1 cells were transiently transfected with murine PPARγ, luciferase-peroxisome proliferator responsive element reporter and β-galactosidase, and treated with *c9t11*-CLA or *t10c12*-CLA. The activation of PPARγ was determined by measuring luciferase activity. By blocking Δ^6 desaturase using the synthetic inhibitor, SC-26196 (44), the ability of CLA isomers to activate PPARγ was reduced ($P < 0.05$). These data indirectly suggest that activation of PPARγ by CLA is increased by the formation of the Δ^6-desaturated products from CLA, *c6c9t11*-CLA or *c6t10c12*-CLA. However, the activation of PPARγ by these products is yet to be measured.

In addition to evidence showing that CLA may induce PPARγ-responsive genes in vivo, CLA may induce the level of PPARγ itself (45). Because PPARγ2 is thought to be one of several transcription factors required for adipose tissue differentiation (41), and because new evidence suggests that activators of PPARγ are protective against cancers arising in the mammary gland, colon and prostate (42), it is possible that some of the molecular mechanisms of action of CLA on carcinogenesis are mediated by PPARγ. Perhaps the ability of PPARγ to mediate effects of CLA is through increased levels of PPARγ protein (45) and/or through activation of PPARγ by downstream metabolites of CLA [e.g., desaturase and elongase products (43)].

In summary, an inverse relationship has been observed between CLA accumulation and outcomes of breast cancer in postmenopausal women (46). However, a preventive role for CLA in human cancer (breast and possibly others) is still unproven. To date, all intervention studies have been conducted in experimental animal models of carcinogenesis. It has been estimated that dietary factors contribute to approximately one third of deaths due to cancer in the United States (47). Because CLA inhibits carcinogenesis in numerous animal models and at multiple stages, this group of fatty acids offers the possibility that several types of cancers in humans

may be prevented with a diet rich in a diversity of chemopreventive compounds, including CLA. More work is required to understand fully the implications of dietary CLA and the possibility of lowering the risk for human cancer development.

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